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ERRATA

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Page 326, line 3: For ref. [1] read Eqn. (1).

Page 327, legends of the two figures should be interchanged.

Page 521, legend Fig. 1: For (a) read (b); for (b) read (a).

BIOCHIMICA ET BIOPHYSICA ACTA, VOL. 36

Page 22, Table I, headings of 3rd and 4th columns: For DPN read TPN and for TPN read DPN.

BIOCHIMICA ET BIOPHYSICA ACTA, VOL. 37

Page 77, ACKNOWLEDGEMENTS: Add: "P.P. is Attaché de Recherches de l'Institut National d'Hygiène, Paris, France, Research Fellow of the Ministère des Affaires Etrangères Francaises (Direction des Relations Culturelles)."

Page 164, legend Table I, between 5th and 6th line insert. "yeast enzyme[6]), 0.18 μmole, $110 \cdot 10^3$ counts/min was added. The methionine vessel included".

Pages 245, 247 and 249, running title: For RNA read DNA.

Pages 484, 486, 488 and 490, running title: For SUOMALEINEN read SUOMALAINEN.

ÉTUDE D'UNE DÉFICIENCE RESPIRATOIRE CHEZ *SACCHAROMYCES CARLSBERGENSIS*

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SUMMARY

Study of a case of deficient respiration in Saccharomyces carlsbergensis

The aerobic growth of *S. carlsbergensis* on a glucose medium is characterized by a phenomenon of diauxy. Without previous adoptation, the glucose, ethanol and acetate respiration of the yeast is feeble. This deficient respiration is adaptive. A kinetic study of the reduction of cytochrome *c*, as well as of the oxidation of the succinate in the extract reveals the site of the deficiency to be at the level of the succinic dehydrogenase. The functioning of the tricarboxylic cycle in *S. carlsbergensis* is considered to be related to the activities obtained for succinic and isocitric dehydrogenases.

INTRODUCTION

La caractérisation biochimique de *S. carlsbergensis* se base essentiellement sur la fermentation du raffinose[1]. Des travaux récents consacrés au métabolisme aérobique des levures de brasserie de fermentation basse, (*S. carlsbergensis*) et des levures de boulangerie (*S. cerevisiae*), semblent indiquer qu'il existe des différences enzymatiques plus profondes entre ces deux races.

Slonimski[2] signale qu'une levure basse utilisant au départ 80 % du glucose par voie fermentaire, ne fermente plus que 14 % du glucose consommé au bout de quelques repiquages en aérobiose.

Elion[3] et Euler[4], ont attribué à la pression d'oxygène au cours de la croissance les différences observées entre levures de brasserie et de boulangerie en ce qui concerne leur intensité de respiration et la structure du spectre des cytochromes.

Fink et Berwald[5] ont décrit la "transformation" d'une levure basse en levure de boulangerie; les conditions de ces expériences ont été critiquées par Slonimski[2].

Tavlitzki et Slonimski[6] émettent l'hypothèse que les levures "basses" pourraient être des lignées dérivées de la levure de boulangerie par le même mécanisme qui conduit à la formation de mutants spontanés[7].

Ces résultats sont en faveur de l'existence d'une déficience enzymatique au niveau de la respiration chez *S. carlsbergensis*.

Alvarez et Wiame[8] ont mis un comportement métabolique analogue en évidence chez *Brettanomyces bruxellensis*. Le faible pouvoir respiratoire de ces levures est attribué à une activité déficiente de l'acéto-CoA-kinase.

SLONIMSKI ET EPHRUSSI[9] après une étude complète des systèmes enzymatiques de la respiration terminale ont montré que, l'incapacité respiratoire des mutants "petites colonies" est principalement due à l'absence de la cytochrome oxydase. SLONIMSKY[2] montre d'autre part, que ces mutants ne possèdent pas la succinique deshydrogénase.

Guidés par ces travaux, nous avons repris le métabolisme de *S. carlsbergensis* par une étude de la croissance aérobique en utilisant une levure de boulangerie et un mutant à déficience respiratoire comme témoin. Ceci nous a permis de mettre en évidence chez *S. carlsbergensis* une déficience respiratoire de nature adaptative. L'analyse des systèmes enzymatiques intervenant dans la respiration a montré que cette déficience se situe au niveau de la succinique deshydrogénase.

MATÉRIEL ET MÉTHODES

Microorganismes: S. cerevisiae: levure Royale (Bruges) isolée en culture pure ainsi que son mutant "petite colonie" isolée d'après EPHRUSSI[7].

S. carlsbergensis: levure de brasserie de fermentation basse isolée en culture pure.

Cultures: Les milieux de culture sont les suivants: milieu glucosé: $(NH_4)_2SO_4$ $2 \cdot 10^{-2}$ *M*; KH_2PO_4 (4 parties) et Na_2HPO_4 (1 partie) de façon à avoir PO_4 10^{-2} *M*; $MgSO_4$ $7H_2O$ $5 \cdot 10^{-3}$ *M*; yeast extract Difco 1 g/l; glucose 1.5 g/l. Milieu éthanol: même milieu mais le glucose est substitué par 0.1 % d'éthanol. Milieu acétate: $(NH_4)_2SO_4$ $2 \cdot 10^{-2}$ *M*; KH_2PO_4 $2 \cdot 10^{-2}$ *M*; $MgSO_4$ $3 \cdot 10^{-2}$ *M*; yeast extract Difco 2.5 g/l; acétate de Na $3H_2O$ 2.7 g/l.

Le pH est ajusté à 4.5 avant stérilisation. Les croissances aérobiques se font à 30° dans des fioles spéciales décrites par WIAME ET STORCK[10]. Les opacités sont exprimées en densité optique (D.O. = log Io/I) pour une épaisseur de 1 cm. L'aération se fait par agitation des fioles sur un plateau animé d'un mouvement de translation circulaire.

Dosage du glucose: Il a été effectué suivant KEILIN ET HARTREE[11]. L'oxygène absorbé est mesuré par la méthode manométrique de Warburg à pH 5.6 et à 30°.

Dosage de l'éthanol: Il a été effectué suivant la méthode de WIDMARCK modifiée par WINNICK'S[12] et CONWAY[13].

Mesures manométriques: Les cellules sont récoltées en phase exponentielle de croissance; ensuite lavées 3 fois par centrifugation dans un tampon KH_2PO_4 *M*/15. L'oxygène absorbée est mesuré par la méthode manométrique de Warburg (v. UMBREIT et coll.[14]). Le volume total de liquide dans les fioles est de 2 ml. Les essais se font à 30° et à pH 4.5.

Préparation des extraits de levure: Après centrifugation et lavage, les levures sont mises en suspension dans un tampon à pH 7.3. Les cellules sont désintégrées dans un oscillateur sonique Raytheon DF/101, 250 W 10 Kc pendant 5 min à — 3°. Après centrifugation (2,500 × *g*) on recueille le surnageant qui constitue l'extrait brut.

Préparation de l'acéto-CoA-kinase: L'enzyme est préparé suivant JONES et Coll.[15] en traitant l'extrait total obtenu après désintégration des cellules, par deux précipitations successives au sulfate d'ammonium à 35 %. Le précipité est recueilli par centrifugation à 18,000 × *g* et remis en suspension dans un tampon phosphate *M*/10 à pH 7.3 (Fraction E).

Dosage de l'acétyl-CoA: Nous utilisons la méthode de TABOR[16]. L'acétyl-CoA formé par l'extrait de levure est transformé en *p*-nitroacétanilide par acétylation enzymatique de la *p*-nitroaniline avec modification du spectre d'absorption à 390 mμ[17].

L'enzyme d'acétylation est préparé à partir de foie de pigeon suivant TABOR[16]. L'unité d'enzyme est défini comme étant la quantité d'enzyme qui cause une chute initiale de la densité optique à 390 mμ de 0.001/min à 25°.

L'activité spécifique est exprimée comme étant le nombre d'unités par mg de protéines.

Analyse des cytochromes: La localisation des bandes d'absorption dues aux cytochromes est déterminée à l'aide d'un spectromètre à déviation constante (Hilger D 186) avec une lampe 30 W. On utilise des suspensions de levures à 25 % (poids humide) dans des cuvettes Unicam (1 cm). Après la mise au point visuelle on photographie les bandes sur plaques Ilford R 40 (rapid process panchromatic). Après révélation on détermine quantitativement les bandes d'absorption à l'aide d'un microphotomètre enregistreur. L'oxydation des cytochromes se fait par barbotage d'oxygène pendant 5 min. Ceci permet la disparition complète de la bande d'absorption à 550 mμ (cytochrome *c*). Cette bande est quantitativement la plus importante et nous servira de repère dans l'étude du système hémoprotéique.

Préparation et dosage de la TPN isocitrique deshydrogénase: L'enzyme est préparé suivant KORNBERG[18]. La fraction contenant la DPN isocitrique deshydrogénase a été éliminée par précipitation au $(NH_4)_2SO_4$. Ensuite à la deuxième précipitation on recueille le précipité dans un tampon phosphate $3 \cdot 10^{-2}$ M à pH 7 (fraction I).

Le DL-isocitrate est préparé à partir de la forme lactone suivant DEUTSCH ET PHILLIPS[19]. L'unité d'enzyme est définie comme étant la quantité d'enzyme qui réduit une μM de TPN en 20 min en utilisant la valeur de $6.22 \cdot 10^6$ cm^2/mole (ouverture de fente 1.6 mμ, chemin optique 1 cm) pour le coefficient d'extinction moléculaire du TPNH[20].

L'activité spécifique est exprimée comme étant le nombre d'unités par mg de protéines.

Dosage des protéines: Il est effectué selon la méthode du biuret. La courbe étalon est établie avec la sérum albumine.

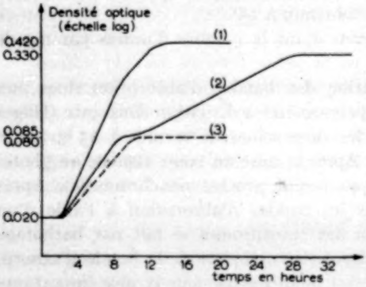
Produits et firmes: Coenzyme A (CoA) California foundation. Adenosine triphosphate (ATP) California foundation. Isocitrate (forme lactone) California foundation. Triphosphopyridine nucleotide (TPN) Sigma.

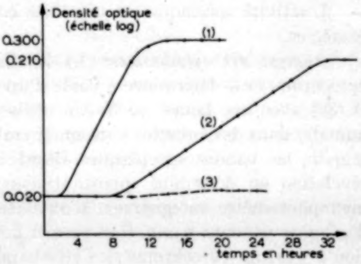
RÉSULTATS

Etude de la croissance aérobique

La Fig. 1 représente la croissance aérobique sur milieu glucosé. Le temps de génération "g" de *S. cerevisiae* est de 1 h 20 min. Le palier indiquant la croissance maximum est situé à une D.O. de 0.420. La courbe de croissance de *S. carlsbergensis* est caractérisé par un phénomène de diauxie. En effet, on observe une première phase exponentielle de croissance avec un "g" de 2 h 20 min; ensuite un arrêt de la croissance pendant 2 h à une D.O. de 0.085; suivi d'une seconde phase exponentielle avec un "g" de 8 h pour atteindre une croissance maximum à une D.O. de 0.330. L'arrêt de croissance après la 1ère phase exponentielle coincide avec la D.O. maximum des mutants.

La Fig. 2 représente la croissance aérobique sur milieu à base d'éthanol ou d'acétate comme substrat carboné. On remarque que *S. carlsbergensis* utilise l'éthanol ou l'acétate avec une phase de latence de 8 h et un "g" de 7 h, alors que *S. cerevisiae* utilise ces mêmes substrats sans adaptation préalable et avec un "g" de 2 h 20 min.





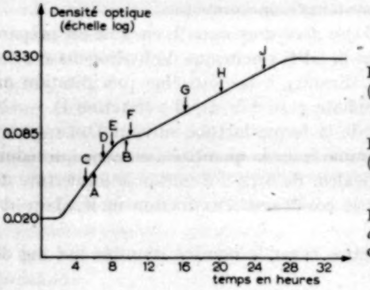


Fig. 1. Croissance aérobique sur 0.15 % de glucose (1) *Saccharomycès cerevisiae*; (2) *Saccharomycès carslbergensis*; (3) mutant respiratoire.

Fig. 2. Croissance aérobique sur 0.1 % d'éthanol, (1) *Saccharomycès cerevisiae*; (2) *Saccharomycès carlbergensis*; (3) mutant respiratoire.

Fig. 3. Croissance aérobique de *Saccharomycès carlsbergensis* sur glucose: A et B: dosage du glucose. C, D, E, F, G, H et J: dosage de l'éthanol.

Dans ces mêmes conditions les mutants ne donnent aucune croissance, ce qui confirme les résultats de TAVLITZKI[21].

Ces résultats nous ont amené à doser le glucose et l'éthanol au cours de la croissance aérobique aux endroits indiqués sur la Fig. 3.

Au départ le milieu de culture contient 8.25 µmoles de glucose/ml; au point A 4.3 µmoles et au point B le glucose est complètement utilisé; au point C nous trouvons 6 µmoles d'éthanol/ml de milieu, en D 10 µmoles; en E 10.7 µmoles; en F 11.5 µmoles; en G 9.2 µmoles; en H 6.2 µmoles et en J l'éthanol est utilisé.

Considérant que 25 % du glucose est utilisé à la synthèse des polysaccharides[22], il reste à la levure 6.24 µmoles de glucose à transformer en éthanol. Dans le cas où *S. carlsbergensis* métabolise le glucose exclusivement par glycolyse fermentative, on devrait trouver à la fin de la 1ère phase exponentielle de croissance 12.48 µmoles d'éthanol. Or au point F, nous obtenons 11.5 µmoles, ce qui vérifie l'hypothèse émise.

Etude du pouvoir respiratoire

La composition en sels minéraux des milieux de culture utilisés pour la récolte des cellules de levure qui serviront aux essais manométriques est la même. La concentration en Yeast Extract est de 0.5 % et les concentrations en substrats carbonés sont de: glucose 4 %; éthanol 1 %; acétate 0.7 %.

Respiration sur glucose, éthanol et acétate après croissance aérobique sur glucose: Les essais sont effectués avec *S. cerevisiae, S. carlsbergensis* et le mutant "petite". Les résultats sont exprimés dans le Tableau I.

TABLEAU I

POUVOIR RESPIRATOIRE DE *S. cerevisiae* ET *S. carlsbergensis* ET D'UN MUTANT "PETITE"

Sur: glucose 4 %, éthanol 60 μM et acétate 60 μM après croissance aérobique sur milieu glucosé 4 % et à 30°.

Souche de levure	Q_{O_2} *endogène*	Q_{O_2} *glucose*	Q_{O_2} *éthanol*	Q_{O_2} *acétate*
S. cerevisiae	7.7	79	103	87
S. carlsbergensis	6.0	16.2	11.1	6.0
Mutant	0.3	0.7	1.2	0.5

TABLEAU II

POUVOIR RESPIRATOIRE DE *S. cerevisiae* ET *S. carlsbergensis*

Sur: glucose 4 %; éthanol 60 μM et acétate 60 μM après croissance aérobique à 30° sur milieu éthanol 1 %.

Souche de levure	Q_{O_2} *endogène*	Q_{O_2} *glucose*	Q_{O_2} *éthanol*	Q_{O_2} *acétate*
S. cerevisiae	11	193	239	234
S. carlsbergensis	21.8	136	178	150

TABLEAU III

POUVOIR RESPIRATOIRE DE *S. cerevisiae* ET *S. carlsbergensis*

Sur: glucose 4 %; éthanol 60 μM et acétate 60 μM après croissance aérobique à 30° sur milieu acétate 0.7 %.

Souche de levure	Q_{O_2} *endogène*	Q_{O_2} *glucose*	Q_{O_2} *éthanol*	Q_{O_2} *acétate*
S. cerevisiae	8.8	223	211.4	226
S. carlsbergensis	15.4	118	115	119

Le faible pouvoir respiratoire de *S. carlsbergensis*, quoique nettement plus élevé que celui des mutants, indique que cette levure utilise le glucose principalement par fermentation. La Fig. 4 représente la cinétique de la respiration du glucose, les valeurs étant toutes rapportées au même poids sec.

Respiration sur glucose, éthanol et acétate apres croissance aérobique sur éthanol et sur acétate: Les essais sont effectués avec *S. cerevisiae* et *S. carlsbergensis*. Les résultats sont exprimés dans les Tableaux II et III.

Nous remarquons une augmentation sensible du pouvoir respiratoire de *S. carlsbergensis* après croissance sur éthanol ou sur acétate comme le montre la Fig. 5.

Les valeurs de la respiration endogène sont également plus élevées, ce qui peut s'expliquer par une accumulation de substrats intermédiaires due au fonctionnement limitant d'un des enzymes de la respiration terminale.

Evolution du pouvoir respiratoire de S. carlsbergensis *au cours d'une croissance aérobique sur milieu glucosé:* Les cellules sont récoltées au cours de la 1ère et de la 2ème phase exponentielle après croissance aérobique sur milieu glucosé 0.15 %. Les résultats sont exprimés dans le Tableau IV.

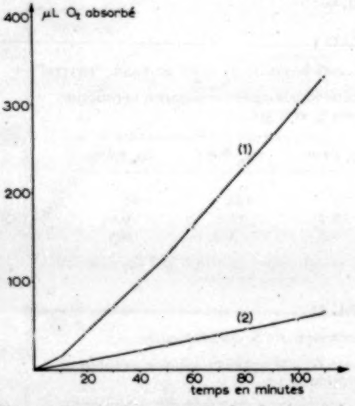


Fig. 4. Respiration sur 4 % de glucose après croissance aérobique sur glucose. Poids sec de levure: 2.5 mg, (1) *Saccharomycès cerevisiae*; (2) *Saccharomycès carlsbergensis.*

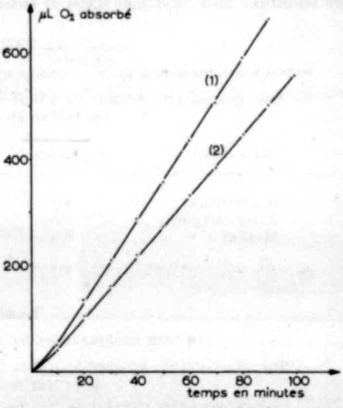


Fig. 5. Respiration sur 4 % de glucose après croissance aérobique sur éthanol. Poids sec de levure: 2.5 mg. (1) *Saccharomycès cerevisiae*; (2) *Saccharomycès carlsbergensis.*

TABLEAU IV

POUVOIR RESPIRATOIRE DE *S. carlsbergensis*

Sur: glucose 4 %; éthanol 60 μM; acétate 60 μM après croissance aérobique sur milieu glucosé 0.15 %; les cellules étant récoltées au cours de la 1ère et de la 2ème phase exponentielle de croissance.

Phase exponentielle de croissance	Q_{O_2} *endogène*	Q_{O_2} *glucose*	Q_{O_2} *éthanol*	Q_{O_2} *acétate*
I	6	16.2	11.1	6
II	14.8	83	90	85

Ceci nous permet de considérer la déficience respiratoire de *S. carlsbergensis* comme étant de nature adaptative.

En effet, en récoltant ces levures au cours de la 2ème phase exponentielle, ou après croissance sur éthanol ou acétate on obtient un pouvoir respiratoire se rapprochant des valeurs obtenues pour *S. cerevisiae.*

Localisation de la déficience respiratoire chez S. carlsbergensis

La mise en évidence d'une déficience respiratoire chez *S. carlsbergensis* nous a amené à étudier la nature enzymatique de cette déficience.

Nous avons admis comme hypothèse de travail que *S. cerevisiae* ainsi que *S. carlsbergensis* après adaptation empruntent le chemin du cycle tricarboxylique pour réaliser leurs oxydations cellulaires[23]. Dans ces conditions le faible pouvoir respiratoire de *S. carlsbergensis* pourrait s'expliquer soit par le freinage provoqué par l'absence d'un des enzymes du cycle de Krebs soit, par le non fonctionnement du système hémoprotéique.

Activité de l'acéto-CoA-kinase: Le témoin est constitué par le système réactionnel complet à l'exception d'un des constituants suivants: enzyme d'acétylation, fraction E ou acétate de K. La concentration en acéto-CoA-kinase (fraction E) est établie de façon qu'elle soit limitante par rapport aux constituants du système complet. Les essais sont effectués avec *S. cerevisiae* et *S. carlsbergensis*. Les résultats sont exprimés dans le Tableau V.

TABLEAU V

ACTIVITÉ DE L'ACÉTO-CoA-KINASE DE *S. cerevisiae* ET *S. carlsbergensis*

Les mesures sont effectuées en cuvettes de 0.6 ml et de 1 cm de chemin optique au spectrophotomètre Unicam à la longueur d'onde de 390 mμ. Le système complet contient: tampon phosphate pH 7.3, 0.05 ml (100 μ*M*); *p*-nitroaniline 0.05 ml (0.05 μ*M*); cystéine 0.05 ml (10 μ*M*); acétate de K 0.05 ml (10 μ*M*); $MgCl_2$ 0.025 ml (5 μ*M*); ATP 0.05 ml (2.5 μ*M*); CoA 0.05 ml (0.1 μ*M*); enzyme d'acétylation 0.075 ml (0.5 mg de protéine); fraction E 0.05 ml (0.09 mg de protéine).

Souche de levure	*Activité en Unités/mg de protéine*	
	Croissance glucose	*Croissance acétate*
S. cerevisiae	342	923
S. carlsbergensis	292	638

Après croissance aérobique sur milieu glucosé, l'activité kinasique de *S. carlsbergensis* est proche de celle de *S. cerevisiae*. Cette activité est fortement accrue après croissance sur acétate.

Description et fonctionnement du système hémoprotéique: Nos mesures spectroscopiques sont effectuées avec *S. cerevisiae* et *S. carlsbergensis* avant et après adaptation à la respiration.

Nous observons les bandes d'absorption suivantes, indépendamment de la souche de levure utilisée: cytochrome *c* à 552 mμ; cytochrome *b* à 560 mμ et cytochrome *a* à 603 mμ.

L'identification des éléments du système hémoprotéique est complétée par l'analyse de la cytochrome oxydase. Cette dernière n'ayant pas de spectre décelable, nous avons utilisé la technique employée par SLONIMSKI[9], qui consiste à oxyder le cytochrome *c* par barbotage d'oxygène dans la suspension de levures. Le fonctionnement normal de la cytochrome oxydase provoque la disparition de la bande d'absorption à 552 mμ [24].

Nous utilisons comme témoin un mutant à déficience respiratoire ne possédant pas la cytochrome oxydase[9]. Ici encore nous n'observons aucune différence entre les 2 races de levures. En effet, le spectre du cytochrome *c* disparaît complètement dans les deux cas après un barbotage d'oxygène de 5 min, alors que dans le cas du mutant on ne constate aucune modification.

Ceci nous a amené a suivre la cinétique de réduction du cytochrome *c* dès l'arrêt du barbotage d'oxygène en photographiant la bande à 552 mμ toutes les 135 sec et en déterminant ensuite après révélation les valeurs d'absorption au microphotomètre enregistreur.

Dans ces conditions les deux races de levures se comportent d'une façon essentiellement différente, comme le montre la Fig. 6.

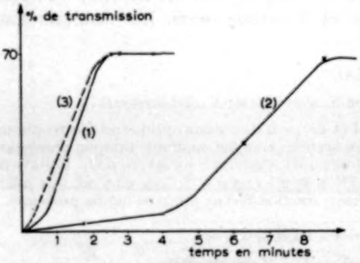


Fig. 6. Cinétique de réduction du cytochrome *c*. 0% de transmission: cytochrome *c* oxydé; 70 % de transmission : cytochrome *c* réduit. (1) *Saccharomycès cerevisiae*; (2) *Saccharomycès carlsbergensis*; (3) *Saccharomycès carlsbergensis* adaptée à la respiration.

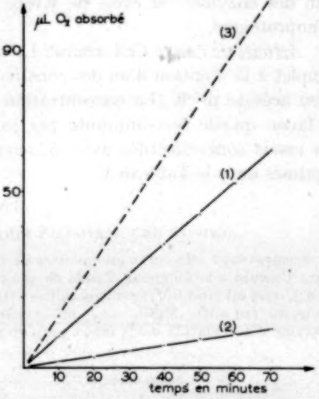


Fig. 7. Oxydation de 10 μM de succinate en extrait (50 mg de protéine) après 12 h d'inanition des différentes levures. (1) *Saccharomycès cerevisiae* ; (2) *Saccharomycès carlsbergensis*; (3) *Saccharomycès carlsbergensis* adaptée à la respiration.

En effet, *S. cerevisiae* réduit complètement le cytochrome *c* 2 min après l'arrêt du barbotage d'oxygène, *S. carlsbergensis* seulement après 8 min. Après croissance sur un substrat respiratoire *S. carlsbergensis* réduit le cytochrome *c* à la même vitesse que *S. cerevisiae.*

Considérant que la réduction du cytochrome *c* dépend principalement de l'activité de la succinique deshydrogénase, nous avons vérifié l'activité de cet enzyme par des essais d'oxydation du succinate '*in vitro*'.

Oxydation du succinate en extrait: Les essais sont effectués avec *S. cerevisiae* et *S. carlsbergensis* après croissance aérobique sur milieu glucosé 2 %. La respiration endogène est réduite par inanition dans un tampon phosphate $M/15$ pH 4.5 pendant 12 h à 30°.

Après centrifugation et lavage, les levures sont mises en suspension dans un tampon triéthanolamine $M/15$ à pH 7.5 contenant 0.5 M lactose[25]. L'extrait total est obtenu après désintégration des cellules et centrifugation.

L'O_2 absorbé est mesuré à 30° par la méthode manométrique de Warburg. Les résultats sont exprimés dans le Tableau VI.

Ces résultats confirment ceux obtenus lors de l'étude de la cinétique de réduction du cytochrome *c*. L'arrêt de la multiplication cellulaire signalé lors de la croissance aérobique (Fig. 1) correspond par conséquent à l'adaptation à la succinique deshydrogénase qui amorce une augmentation proportionnelle du pouvoir respiratoire comme le montre le Tableau VII.

TABLEAU VI

OXYDATION DU SUCCINATE (10 μM) EN EXTRAIT PAR *S. cerevisiae* ET *S. carlsbergensis*

Souche de levure	*Mg de protéine de l'extrait*	*Temps d'incubation en minutes*	*µl O_2 absorbé*	
			endogène	*succinate*
S. cerevisiae	61	60	14	68
		120	30	109
S. carlsbergensis	52	60	4	12
		120	8	15
S. carlsbergensis adapté	50	60	11	91
		120	17	148

TABLEAU VII

OXYDATION DU SUCCINATE ET POUVOIR RESPIRATOIRE DE *S. carlsbergensis* AVANT ET APRÈS ADAPTATION À LA RESPIRATION

Souche de levure	*Avant adaptation*		*Après adaptation*	
	µl O_2 absorbé succinate	Q_{O_2}	*µlO_2 absorbé succinate*	Q_{O_2}
S. carlsbergensis	15	16	148	136

TABLEAU VIII

ACTIVITÉS DE LA TPN ISOCITRIQUE DESHYDROGÉNASE CHEZ *S. cerevisiae* ET *S. carlsbergensis*

Les mesures sont effectuées en cuvettes de quartz de 4 ml et de 1 cm de chemin optique au spectrophotomètre Zeiss PMQ II à 340 mμ. Le système complet contient: tampon phosphate pH 7 0.2 ml (100 μM); $MgCl_2$ 0.1 ml (10 μM); TPN 0.02 ml (0.5 μM); isocitrate 0.1 ml (0.5 μM); fraction I 1.0 ml (0.1 mg de protéine).

Souche de levure	*Activité en Unités/mg protéines*
S. cerevisiae	0.53
S. carlsbergensis non adapté	0.26
S. carlsbergensis adapté	1.57

Activité de l'isocitrique deshydrogénase: Nous nous sommes demandés si la succinique deshydrogénase était la seule deshydrogénase responsable de la déficience respiratoire de *S. carlsbergensis*.

Les activités de la TPN isocitrique deshydrogénase sont déterminées chez *S. cerevisiae* et *S. carlsbergensis* avant et après adaptation à la respiration. Les levures sont récoltées après croissance aérobique sur milieu glucosé à 2 %.

Le témoin est constitué par le système réactionnel complet sans isocitrate. La concentration en isocitrique deshydrogénase (fraction I) est établie de façon qu'elle soit limitante par rapport à la concentration en TPN et en isocitrate. Les résultats sont exprimés dans le Tableau VIII.

Ces résultats indiquent que la respiration de *S. carlsbergensis* est également freinée par l'activité de l'isocitrique deshydrogénase avant l'adaptation à la respiration. L'activité spécifique augmente environ 6 fois après adaptation.

DISCUSSION

Des résultats décrits dans ce travail, il résulte que le métabolisme aérobique de *S. carlsbergensis* est caractérisé par un phénomène de diauxie. En effet, dans des conditions ou la croissance est limitée par la concentration en substrat carboné, cette levure utilise le glucose par fermentation avec accumulation d'éthanol qu'elle utilise ensuite après adaptation.

D'autre part, la vitesse de croissance sur éthanol ou acétate est la même que celle observée lors de la seconde phase exponentielle de croissance. Ces substrats ne sont utilisés qu'après une phase d'adaptation préalable explicitée par une phase de latence de 6 à 8 h.

La détermination des pouvoirs respiratoires dans les différentes conditions de croissance confirme les résultats précédents. *S. carlsbergensis* respire faiblement le glucose, l'éthanol et l'acétate au cours de la 1ère phase exponentielle de croissance. Les valeurs sont cependant supérieures à celles obtenues pour les mutants.

Au cours de la seconde phase exponentielle de croissance ou après croissance sur éthanol ou acétate, le pouvoir respiratoire est rétabli et se rapproche de celui de *S. cerevisiae*.

De ce qui précède, nous pouvons déjà observer que la déficience respiratoire de *S. carlsbergensis* est de nature adaptative.

Les travaux de LINNANE ET STILL[26] et de VANDERWINKEL, BOURGEOIS ET WIAME[25] ont montré que le cycle des acides tricarboxyliques existe chez la levure et constitue probablement la voie essentielle d'oxydation.

Ceci nous a amené à admettre comme hypothèse de travail que *S. cerevisiae* ainsi que *S. carlsbergensis* après adaptation à la respiration empruntent le cycle tricarboxylique pour réaliser leurs oxydations cellulaires.

Vu l'analogie avec le comportement métabolique de *Brettanomyces bruxellensis*[8] nous avons vérifié l'activité de l'acéto-CoA-kinase. Nous avons obtenu une activité kinasique normale chez *S. carlsbergensis* avant adaptation à la respiration ou même après croissance anaérobique.

Le fonctionnement de cet enzyme chez *S. carlsbergensis* permet d'envisager la synthèse des acides gras saturés. L'analyse spectroscopique du système hémoprotéique a montré l'existence des cytochromes *a*, *b*, *c* et de la cytochrome oxydase, indépendamment de la souche utilisée.

En suivant la cinétique de réduction du cytochrome *c* nous avons remarqué que la vitesse d'apparition du spectre du cytochrome *c* après oxydation est beaucoup plus rapide dans le cas de *S. cerevisiae*. Cette différence disparaît après croissance sur un substrat respiratoire.

Ceci nous permet de délimiter la déficience respiratoire de *S. carlsbergensis* au niveau du succinate. Ce point de vue a été entièrement confirmé par des essais d'oxydation du succinate *in vitro*.

En effet, l'adaptation à la succinique deshydrogénase se fait proportionnellement à l'adaptation du pouvoir respiratoire des cellules entières.

En examinant les valeurs des potentiels propres à chacune des deshydrogénations, WIAME[23] fait remarquer que la deshydrogénation succinique a le potentiel le plus élevé. ($E_o' = 0{,}00$) L'absence de la succinique deshydrogénase chez *S. carlsbergensis* n'exclut donc pas nécessairement le fonctionnement des autres deshydrogénases du cycle. Le rôle énergétique et synthétique du cycle tricarboxylique en anaéobiose ne semble d'ailleurs pas exclu[23]. Aubert et Milhaud[27] arrivent à la même conclusion en mettant des intermédiaires du cycle de Krebs en évidence après fermentation.

Nous avons essayé de résoudre ce point en déterminant l'activité de l'isocitrique deshydrogénase.

Avant l'adaptation à la respiration nous remarquons une activité nettement supérieure à celle de la succinique deshydrogénase. Ceci semble mettre en évidence un fonctionnement partiel du cycle tricarboxylique chez *S. carlsbergensis* non adapté.

L'inversion des réactions enzymatiques avec accumulation de métabolites intermédiaires jusqu'au succinate, nous parait justifier la valeur trouvée pour l'activité de l'isocitrique deshydrogénase.

RÉSUMÉ

La croissance aérobique de *S. carlsbergensis* sur milieu glucosé est caractérisée par un phénomène de diauxie. Cette levure respire faiblement le glucose, l'éthanol et l'acétate sans adaptation préalable. La déficience respiratoire est de nature adaptative. L'étude de la cinétique de réduction du cytochrome *c*, ainsi que de l'oxydation du succinate en extrait permet de localiser cette déficience au niveau de la succinique deshydrogénase. Le fonctionnement du cycle tricarboxylique chez *S. carlsbergensis* est considéré en fonction des activités obtenues pour la succinique deshydrogénase et l'isocitrique deshydrogénase.

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PHOSPHORYLATION COUPLED TO ELECTRON TRANSPORT MEDIATED BY HIGH POTENTIAL ELECTRON CARRIERS

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SUMMARY

1. It is shown that with fresh preparations of rat liver mitochondria, phosphorylation coupled to electron transport to oxygen initiated by exogenous cytochrome *c*, silicomolybdate or ferricyanide, maintained reduced with ascorbate, requires the presence of factors which apparently act to stabilize the mitochondrial structure. These factors are not necessary for obtaining maximal P/O ratios in succinate oxidation. The requirements are a mildly alkaline reaction medium (pH 7.8–8.0), fluoride ($7.5 \cdot 10^{-3}\,M$), EDTA ($3 \cdot 10^{-4}\,M$) or phosphotungstate ($7.5 \cdot 10^{-6}\,M$) with cytochrome *c*; Ba^{++}, Sr^{++} or Mn^{++} ($2 \cdot 10^{-4}\,M$) or Mg^{++} ($7.5 \cdot 10^{-3}\,M$) with silicomolybdate; and Ba^{++} or Sr^{++} ($2 \cdot 10^{-4}\,M$) or Mg^{++} ($7.5 \cdot 10^{-3}\,M$) with ferricyanide. The P/O ratios obtained with silicomolybdate and ferricyanide lie between 1.0 and 2.0, depending on the concentration of the complex anion.

2. It is shown that silicomolybdate can also mediate the oxidation of external DPNH by intact rat-liver mitochondria and that phosphorylation is very efficiently coupled to this oxidation in the absence of the special divalent metal ions which are required when ascorbate is used to maintain the silicomolybdate in its reduced state. Ferrocyanide is also capable of mediating DPNH oxidation but only in the presence of high ($7.5 \cdot 10^{-3}\,M$) concentrations of Mg^{++}. Phosphorylation is efficiently coupled to this oxidation. The high potential electron donors initiate electron transport reactions more efficiently when DPNH rather than ascorbate is used to maintain the donors reduced. It is suggested that DPNH modifies the permeability of the mitochondrial surface.

3. It is suggested that the data generally support the concept that electron transport initiated by high potential electron carriers disrupts some structural feature of the mitochondria which maintains the coupling between respiration and phosphorylation and that special ionic factors can stabilize the system against such disruption.

INTRODUCTION

Phosphorylation coupled to electron transfer from exogenous ferrocytochrome *c* to oxygen catalyzed by the respiratory enzyme complex of mitochondria has been

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demonstrated by several workers[1–8]. However, the experimental conditions reported to be necessary for efficient coupling have been varied and somewhat incompatible. For example, Lehninger *et al.*[5] found that a preliminary exposure of the mitochondria to hypotonic media and a low ($1 \cdot 10^{-5}$ *M*) concentration of exogenous cytochrome *c* in the reaction mixture were necessary to approach P/O ratios of 1.0, whereas Maley and Lardy[4] used only isotonic sucrose and a 10-fold greater concentration of cytochrome *c* and found that the critical requirements were low levels of ascorbate and mitochondria. We have examined in greater detail the electron transport reactions initiated by exogenous ferrocytochrome *c* and found that a number of structurally unrelated anions (fluoride, EDTA, phosphotungstate, hydroxyl) produced significant and sometimes large increases in their P/O ratios. These same anions were not required to obtain maximal phosphorylation with succinate oxidation. It is interesting that fluoride and EDTA had been used routinely in previous work[1–8].

During the course of these studies it was found that the complex anions silico-12 molybdate[53] and ferrocyanide, both of which form high potential reversible oxidation–reduction couples, could be used in the place of exogenous cytochrome *c* to mediate electron transport to oxygen in the respiratory enzyme chain of mitochondria. In order to obtain phosphorylation coupled to these oxidations it was again necessary to supplement the reaction mixture with special factors (the divalent metal ions Mg^{++}, Ba^{++}, Sr^{++} or Mn^{++}) which were not required to obtain maximal phosphorylation with succinate oxidation. Glenn and Crane[6] have previously shown that the oxidation of aldehydes by the soluble aldehyde oxidase flavoprotein can be coupled to cytochrome *c* reduction with the aid of silicomolybdate. Their observations initially led us to explore the interaction of silicomolybdate with the mitochondrial electron transport chain.

In most of the above-mentioned experiments substrate quantities of ascorbate have been employed to maintain catalytic amounts of the high potential electron carriers in the reduced state during the course of the reactions. When ascorbate was replaced with exogenous DPNH it was found that the reaction pattern exhibited striking changes. In this paper we describe some characteristics of the reactions obtained when cytochrome *c*, silico-12 molybdate and ferricyanide were used to mediate the oxidations of ascorbate or DPNH by rat-liver mitochondria. The implications of the results on a relationship between mitochondrial structure and function are discussed. The preliminary experiments which have been developed in this paper were published earlier[9].

EXPERIMENTAL

The livers of 150–250 g male rats were ground in 0.3 *M* sucrose with a Teflon-glass homogenizer and the mitochondrial fraction was obtained by differential centrifugation essentially as described in the literature[10, 11]. For experiments with succinate oxidation the flasks contained a basic reaction mixture defined as potassium succinate ($1.5 \cdot 10^{-2}$ *M*), potassium phosphate ($1 \cdot 10^{-2}$ *M*, pH 7.0), ATP ($1.5 \cdot 10^{-3}$ *M*), glucose ($6 \cdot 10^{-2}$ *M*), sucrose (0.3 *M*), $MgCl_2$ ($1.5 \cdot 10^{-3}$ *M*), hexokinase (0.5 mg) and freshly prepared rat-liver mitochondria (8–10 mg protein). To this was added the special components indicated in the tables or legends to the respective figures. The final volume was 3.3 ml. For experiments with ascorbate or DPNH as substrates the flasks contained a basic reaction mixture defined as potassium ascorbate ($7.5 \cdot 10^{-3}$ M) or DPNH ($6 \cdot 10^{-3}$ M) respectively, potassium phosphate ($7.5 \cdot 10^{-3}$ M, pH 7.0), glucose ($6 \cdot 10^{-2}$ M), sucrose (0.3 M), hexokinase (0.5 mg) and freshly prepared rat-liver mitochondria (8–10 mg protein). To this was added the ATP, divalent metal ions, cytochrome *c*, silicomolybdate, potassium ferricyanide and other components, as indicated in the tables or figures. The final reaction volume was 3.3 ml. The center wells of all flasks contained 0.2 ml 5 N KOH. Oxygen consumption, for a period indicated in the text, was measured in a Warburg apparatus at 30° with air as the gas phase. The flasks were equilibrated in the bath for the first 6 min and it was assumed that the reaction during this period proceeded at the same rate as for the following 6 min. The reaction was terminated by addition of 1 ml 15% trichloroacetic acid. Inorganic phosphate in the supernatant was measured by the method of FISKE AND SUBBAROW[12]. Calibration of the assay was carried out in the presence of various quantities of ascorbate in order to include the small absorbancy correction at 750 mμ introduced by the unoxidized ascorbate remaining in the flasks at the end of the experiment. The presence of silicomolybdate in the experimental flasks, in the concentrations used in these experiments, did not significantly interfere with phosphate determinations by the FISKE AND SUBBAROW method. The presence of ferrocyanide, in concentrations up to $1.5 \cdot 10^{-3}$ M, also did not significantly interfere with the inorganic phosphate determinations. However, in this case, a bluish-black, rather than pure blue, color developed upon addition of reducing agent but the black component contributed only very slightly to the optical absorption at 750 mμ. Absorbancy measurements at 750 mμ were converted to phosphate concentrations by their comparison with standard curves obtained with known amounts of phosphate and ferrocyanide. With phosphate concentrations measured in this way, the accuracy of the computed P/O ratio was better than 95%. When larger concentrations (up to $1.8 \cdot 10^{-2}$ M) of ferrocyanide were present in the experimental flasks, inorganic phosphate was determined by the same general assay method. At these higher concentrations, however, a flocculent precipitate formed upon addition of the reducing agent. This was separated from the clear blue supernatant solution after 10 min of color development, by centrifugation. Absorbancy measurements of the clarified supernatant were again converted to phosphate concentrations by comparison with an appropriate set of standard absorption curves. Care must be taken to use freshly prepared reducing solution or a colloidal suspension, difficult to clarify by centrifugation, will form. Flocculation of the precipitate was aided by carrying out the reaction in 0.5 M NaCl although this procedure was not adopted routinely. With phosphate concentrations measured in this way, the accuracy of the computed P/O ratio was better than 90%.

For calculation of all P/O ratios, both inorganic phosphate disappearance and oxygen consumption were computed relative to their values observed in control flasks which contained all of the reaction components except the cytochrome *c*, silicomolybdate or ferricyanide. Oxygen uptake in the control flasks was usually less than 10% of that observed in the experimental flask with the slowest reaction rate. Inorganic phosphate remained essentially constant in the control flasks during the experiment.

The hexokinase preparation was purified through step 3a according to the procedure of BERGER *et al.*[13]. Its activity was assayed with 20 μmoles Tris buffer, pH 7.5, 25 µmoles $MgCl_2$, 5 µmoles ATP and 200 µmoles glucose in 3 ml volume at 30°. Under these conditions 0.05 mg of the preparation catalyzed the transfer of 2 µmoles phosphate in 10 min. Protein determinations were made with the biuret reagent[14] after addition to the mitochondrial sample of an equal volume of 5% deoxycholate. Antimycin A was assayed by its absorption at 320 mµ in ethanol[15]. Cytochrome *c* (horse heart) was purchased from Sigma Chemical Co. and purified on Amberlite XE-97 cation-exchange resin according to the procedure of MARGOLIASH[16]. Crystalline ascorbic acid, supplied by Merck and Co. was neutralized with KOH immediately before use. DPNH, prepared by enzymic reduction of DPN, was obtained from Sigma Chemical Co., and used without additional purification.

A solution of $1 \cdot 10^{-3}$ *M* silicomolybdate[53] was prepared by dissolving 14.4 mg sodium metasilicate and 120 mg sodium molybdate in 50 ml water containing 0.4 ml 5 *N* HCl and used within 30–60 min. Immediately before use the pH was adjusted to 5.5 with KOH.

RESULTS

With succinate as substrate, standard aliquots (8–10 mg protein) of freshly prepared rat-liver mitochondria used in these experiments consumed 12–15 µatoms oxygen in 18 min and gave satisfactory P/O ratios (between 1.5–1.9) in the unsupplemented basic reaction mixture previously defined. Occasional preparations were obtained which did not give P/O ratios greater than 1.3 unless the basic reaction mixture was either brought to pH 7.5–7.7 or supplemented with additional magnesium (Table I). Such preparations were routinely discarded. The oxidation of ascorbate by the mitochondria in the absence of supplementary electron carriers proceeded extremely

TABLE I

EFFECT OF VARIOUS ADDITIONS ON PHOSPHORYLATION COUPLED TO SUCCINATE OXIDATION

Electron carrier	*Additions Mg ($M \times 10^3$)*	*Other*	*Oxygen µatoms 18 min*	*P/O*
—	1.5	—	15.1	1.33
—	7.5	—	13.5	1.67
—	1.5	pH 7.6*	14.5	1.69
—	1.5	—	14.9	1.61
—	7.5	—	13.9	1.69
—	1.5	Ascorbate ($7.5 \cdot 10^{-3}$ *M*)	14.8	1.59
Cytochrome *c* ($2 \cdot 10^{-5}$ *M*)	7.5		13.5	1.65
Cytochrome *c* ($2 \cdot 10^{-5}$ *M*)	7.5	Fluoride ($7.5 \cdot 10^{-3}$ *M*)	13.1	1.66
Cytochrome *c* ($2 \cdot 10^{-5}$ *M*)	7.5	EDTA ($3 \cdot 10^{-4}$ *M*)	14.0	1.60
Silicomolybdate ($6 \cdot 10^{-5}$ *M*)	1.5	—	14.4	1.51
Silicomolybdate ($6 \cdot 10^{-5}$ *M*)	7.5	—	13.5	1.55
Ferrocyanide ($3 \cdot 10^{-3}$ *M*)	1.5	—	14.5	1.35
Ferrocyanide ($3 \cdot 10^{-3}$ *M*)	7.5	—	13.8*	1.65
Silicomolybdate ($6 \cdot 10^{-5}$ *M*)	1.5	—	14.9	1.55
Silicomolybdate ($6 \cdot 10^{-5}$ *M*)	7.5	—	14.6	1.40
Ferrocyanide ($3 \cdot 10^{-3}$ *M*)	1.5	—	15.1	1.59
Ferrocyanide ($3 \cdot 10^{-3}$ *M*)	7.5	—	14.5	1.60

* pH of reaction mixture.

slowly. Oxygen consumption could be mediated by the addition of either cytochrome *c*, silicomolybdate or ferricyanide. The oxidation rates at infinite ascorbate concentration[17] were about twice those obtained with $7.5 \cdot 10^{-3}\ M$ ascorbate. The lower concentration of ascorbate usually gave slightly higher P/O ratios, in agreement with the observations of MALEY AND LARDY[4]. Both the oxidation rates and P/O ratios were additionally dependent on the presence of special factors in the basic reaction mixture. These phenomena are described for each electron donor.

Cytochrome c

When catalytic amounts of cytochrome *c* were added to the basic reaction mixture containing ascorbate, esterification of inorganic phosphate was not coupled to the subsequent electron transport to molecular oxygen unless the reaction mixture was further supplemented with either EDTA at $3 \cdot 10^{-4}\ M$, fluoride at $7.5 \cdot 10^{-3}\ M$ or phosphotungstate at $7.5 \cdot 10^{-6}\ M$ (Table II). High concentrations of Mg^{++} ($7.5 \cdot 10^{-3}\ M$) were necessary to demonstrate the effect of fluoride but not that of EDTA. It was also found that both the oxidation rates and P/O ratios were higher in a mildly alkaline reaction medium containing $7.5 \cdot 10^{-3}\ M$ Mg^{++} (Fig. 1 and Table II). Neither fluoride, EDTA, phosphotungstate, cytochrome *c* nor ascorbate, in the concentrations used in these experiments, had any effect on the P/O ratios accompanying succinate oxidation (Table I).

TABLE II

EFFECT OF VARIOUS ANIONS ON PHOSPHORYLATION COUPLED TO ASCORBATE-CYTOCHROME *c* OXIDATION

Anion	*Oxygen µatoms 24 min*	*P/O*
—	8.1	0.00
Fluoride ($7.5 \cdot 10^{-3}\ M$)	6.1	0.66
EDTA ($3 \cdot 10^{-4}\ M$)	6.0	0.86
PTA* ($7.5 \cdot 10^{-6}\ M$)	5.9	0.35
OH (pH 8.0)	10.9	0.91
OH (pH 8.0)**	11.5	1.01
—	7.3	0.10
Fluoride ($7.5 \cdot 10^{-3}\ M$)	5.1	0.58
EDTA ($3 \cdot 10^{-4}\ M$)	5.8	0.91
PTA* ($7.5 \cdot 10^{-6}\ M$)	5.0	0.53
OH (pH 7.8)**	9.8	1.10

In addition to basic reaction mixture for experiments with ascorbate as described in text, all flasks contained $7.5 \cdot 10^{-3}\ M$ $MgCl_2$, $2 \cdot 10^{-5}\ M$ cytochrome *c* and $1.5 \cdot 10^{-3}\ M$ ATP. Additions are indicated in Table.

* Added directly to the mitochondria from an unneutralized solution of phosphotungstic acid before addition of any reaction components.

** Buffered with $7.5 \cdot 10^{-3}\ M$ Tris.

We were able to confirm the observations of LEHNINGER *et al.*[5] that the P/O ratios increase as the cytochrome *c* concentration is decreased. We found this to be true with all of the factors which supported coupled phosphorylation. In their experiments the highest P/O ratios achieved were somewhat less than 1.0. However, in some of our experiments carried out in the pH range 7.8–8.0 with $1 \cdot 10^{-5}\ M$

cytochrome *c*, P/O ratios significantly above 1.0 were obtained (Fig. 1). Combination of two or more factors occasionally improved P/O ratios but no attempt was made to find optimal combinations.

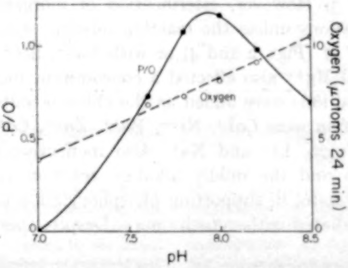

Fig. 1. Effect of pH on oxygen consumption and coupled phosphorylation mediated by exogenous cytochrome *c*. All flasks contained the basic reaction mixture for experiments with ascorbate ($7.5 \cdot 10^{-3}$ *M*) as described in the text and $7.5 \cdot 10^{-3}$ *M* Tris buffer, and $7.5 \cdot 10^{-3}$ *M* Mg^{++}.

It was found that pH effects similar to those described above could be observed under still other conditions. With mitochondrial preparations which had been aged in 0.3 *M* sucrose at 0°, phosphorylation was more efficiently coupled to succinate oxidation in mildly alkaline reaction media (Table III). For these experiments the reaction mixture was adjusted to the indicated pH by the addition of KOH. A supplementary buffer (Tris) was omitted in these experiments in order to demonstrate that the effect is primarily due to H^+ concentration. The data indicate that optimal effect is obtained close to pH 8.5.

TABLE III

EFFECTS OF VARIOUS ADDITIONS ON PHOSPHORYLATION COUPLED TO SUCCINATE OXIDATION IN AGED MITOCHONDRIA

Age h at 0°	*Additions*	*pH**	*Oxygen μatoms 18 min*	*P/O*
0	—	7.0	13.5	1.67
0	—	8.5	13.5	1.10
0	$MgCl_2$ ($7.5 \cdot 10^{-3}$ *M*)	7.0	13.0	1.69
24	—	7.0	15.9	0.39
24	$MgCl_2$ ($7.5 \cdot 10^{-3}$ *M*)	7.0	14.0	1.22
48	$MgCl_2$ ($7.5 \cdot 10^{-3}$ *M*)	7.0	16.9	0.10
48	$MgCl_2$ + $2 \cdot 10^{-4}$ *M* $MnCl_2$	7.0	13.9	0.83
72	$MgCl_2$ + $2 \cdot 10^{-4}$ *M* $MnCl_2$	7.0	16.1	0.00
72	$MgCl_2$ ($7.5 \cdot 10^{-3}$ *M*)	8.0	16.5	0.53
72	$MgCl_2$ ($7.5 \cdot 10^{-3}$ *M*)	8.5	15.8	0.85
72	$MgCl_2$ ($7.5 \cdot 10^{-3}$ *M*)	9.0	15.2	0.31
72	$MgCl_2$ ($7.5 \cdot 10^{-3}$ *M*)	7.0	18.2	0.00
72	$MgCl_2$ ($7.5 \cdot 10^{-3}$ *M*)	8.0	17.1	0.30
72	$MgCl_2$ ($7.5 \cdot 10^{-3}$ *M*)	8.5	17.5	0.53
72	$MgCl_2$ ($7.5 \cdot 10^{-3}$ *M*)	9.0	17.0	0.40

* pH of reaction mixture.

Silicomolybdate

The marked enhancement of oxygen consumption upon addition of catalytic amounts of silicomolybdate to the basic reaction mixture containing ascorbate is shown in Figs. 2 and 3. However, esterification of inorganic phosphate was not coupled to these oxidations unless the reaction mixture was further supplemented with Mg^{++} at $7.5 \cdot 10^{-3}$ *M* (Figs. 2 and 4) or with Ba^{++}, Sr^{++} or Mn^{++} at $2 \cdot 10^{-4}$ *M* (Fig. 5). The additional Mg^{++} also effected a concomitant increase in the oxidation rates (Fig. 2). All metal ions were added as the chloride salts. Ineffective at either low or high concentration were Co^{++}, Ni^{++}, Be^{++}, Zn^{++}, Cd^{++}, Hg^{++}, Pb^{++}, Ca^{++}, Cu^{++}, Fe^{++}, Al^{+++}, Fe^{+++}, Li^{+} and Na^{+}. Also ineffective were fluoride, EDTA, bovine serum albumin and the mildly alkaline reaction conditions (pH 7.5-8.0) which had proved successful in supporting phosphorylation coupled to the electron transport reactions mediated with cytochrome *c*. Despite these striking results with ascorbate-silicomolybdate, neither Mg^{++} at $7.5 \cdot 10^{-3}$ *M* nor Ba^{++}, Sr^{++} or Mn^{++} at $2 \cdot 10^{-4}$ *M* had any significant effect on the P/O ratios obtained with succinate oxidation in fresh mitochondria. Fig. 4 compares the effect of Mg^{++} concentration on the P/O ratios accompanying the oxidations of succinate and silicomolybdate. In the experiments shown in Figs. 3 and 5, all flasks contained Mg^{++} at a concentration of $1.5 \cdot 10^{-3}$ *M* in addition to the specified amounts of Ba^{++}, Sr^{++}, or Mn^{++}. It can be seen from Fig. 4 that this low concentration of Mg^{++} maintained maximal P/O ratios with succinate oxidation but could not alone effectively support phosphorylation coupled to silicomolybdate oxidation. Mitochondria which had been aged for 24 h in 0.3 *M* sucrose at 0° did, however, require higher levels of Mg^{++} for maximal phosphorylation coupled to succinate oxidation (Table II). Low levels of Mn^{++} had a similar effect on mitochondria which had been so aged for 48 h (Table II). In experiments with aged mitochondria, centrifugation following the aging period was avoided since it was found that the P/O ratios obtained with preparations aged for only 24 h became extremely sensitive to centrifugal forces.

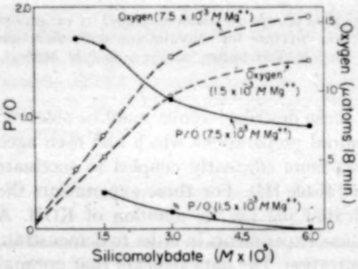

Fig. 2. Effect of $7.5 \cdot 10^{-3}$ *M* Mg^{++} on oxygen consumption and coupled phosphorylation mediated by silicomolybdate. All flasks contained the basic reaction mixture for experiments with ascorbate ($7.5 \cdot 10^{-3}$ *M*) as described in the text and the additional components indicated in the Figure.

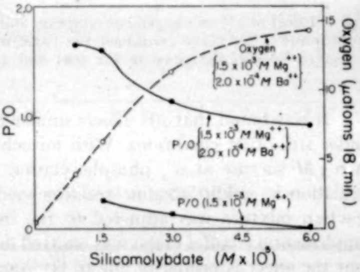

Fig. 3. Effect of $2 \cdot 10^{-4}$ *M* Ba^{++} on oxygen consumption and coupled phosphorylation mediated by silicomolybdate. Other reaction conditions are identical with those of Fig. 2.

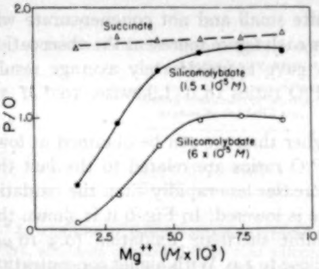

Fig. 4. Effect of Mg^{++} concentration on phosphorylation coupled to electron transport mediated by silicomolybdate. The lack of effect of Mg^{++} on phosphorylation coupled to succinate oxidation is also shown (dashed curve). Other reaction conditions are identical with those of Fig. 2.

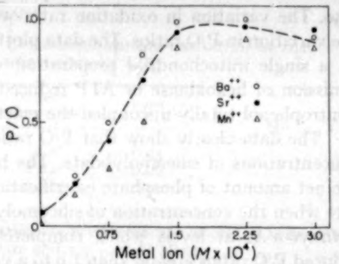

Fig. 5. Effect of Ba^{++}, Sr^{++} and Mn^{++} concentrations on phosphorylation coupled to electron transport mediated by $6 \cdot 10^{-5}$ *M* silicomolybdate. All flasks contained $1.5 \cdot 10^{-3}$ *M* Mg^{++} in addition to the divalent metal ions indicated in the figure. Other reaction conditions are identical with those of Fig. 2.

The maximum possible P/O ratio which could be attained with a single concentration of silicomolybdate was dependent on the concentration of ATP (Table IV) and to a much lesser extent on the level of hexokinase. In the experiments described in this paper, hexokinase was maintained at the level indicated in the experimental section and the optimal ATP:silicomolybdate ratios were chosen from the data of Table IV.

TABLE IV

EFFECT OF ATP CONCENTRATION ON P/O RATIOS FOR ASCORBATE–SILICOMOLYBDATE OXIDATION

Metal ion *(M × 10^3)*	*ATP* *(M × 10^3)*	*Silicomolybdate*		
		$1.5 \cdot 10^{-5}$ M	*$3.0 \cdot 10^{-5}$ M*	*$6.0 \cdot 10^{-5}$ M*
Mg^{++} (7.5)	0.0	0.0	0.0	0.0
	0.15	1.61	—	—
	0.30	2.12	0.77	0.30
	0.75	1.50	1.20	0.67
	1.5	1.13	1.10	0.96
	3.0	—	—	0.71
Mg^{++} (1.5) + Ba^{++} (0.2)	0.0	0.0	0.0	0.0
	0.15	1.43	—	0.21
	0.30	1.78	1.05	0.45
	0.75	1.60	1.25	0.87
	1.5	1.43	1.01	0.99
	3.0	—	—	0.80

There was some variation in the P/O ratios obtained with different mitochondrial preparations. The variation was minimal under those experimental conditions giving P/O ratios close to 1.0. For 25 mitochondrial preparations assayed with $6 \cdot 10^{-5}$ *M* silicomolybdate and $7.5 \cdot 10^{-3}$ *M* Mg^{++}, the average P/O ratio was 0.90 with a spread between 0.79 and 1.21. For 15 preparations assayed with $1.5 \cdot 10^{-5}$ *M* silicomolybdate and $7.5 \cdot 10^{-3}$ *M* Mg^{++}, the average P/O ratio was 1.49 with a spread between 1.25 and

2.50. The variation in oxidation rates was quite small and not commensurate with the variation in P/O ratios. The data plotted for each figure represent the observations on a single mitochondrial preparation which gave approximately average results. Omission of hexokinase or ATP reduced all P/O ratios to 0. Likewise, 10^{-4} *M* 2,4-dinitrophenol totally uncoupled the reactions.

The data clearly show that P/O ratios higher than 1.0 can be obtained at lower concentrations of silicomolybdate. The high P/O ratios are related to the fact that the net amount of phosphate esterification decreases less rapidly than the oxidation rate when the concentration of silicomolybdate is lowered. In Fig. 6 it is shown that antimycin A, at levels which completely inhibit succinate oxidation (0.3–10 μg), reduced P/O ratios greater than 1.0 to a value close to 1.0. With higher concentrations of silicomolybdate, where the P/O ratios were no higher than 1.0, antimycin A had no effect at the above levels (Fig. 7). The complete inhibition of phosphorylation at much higher levels of antimycin, seen in Figs. 6 and 7, has also been observed by NIELSEN AND LEHNINGER[6] with phosphorylation coupled to the oxidation of ferrocytochrome *c*.

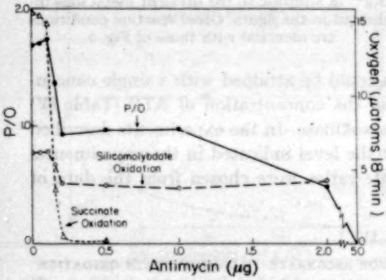

Fig. 6. Effect of antimycin A on oxygen consumption and coupled phosphorylation mediated by $1.5 \cdot 10^{-5}$ *M* silicomolybdate. The effect of antimycin A on succinate oxidation is also shown (△—△). All flasks contained $7.5 \cdot 10^{-3}$ *M* Mg^{++}. Other reaction conditions are identical with those of Fig. 2.

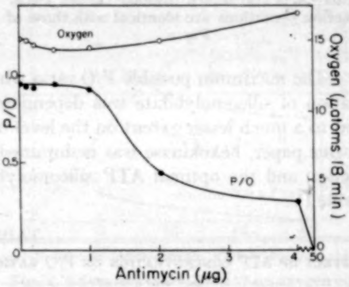

Fig. 7. Effect of antimycin A on oxygen consumption and coupled phosphorylation mediated by $6 \cdot 10^{-5}$ *M* silicomolybdate. Other reaction conditions are identical with those of Fig. 6.

The data of Table I demonstrate that phosphorylation coupled to succinate oxidation was not significantly inhibited by silicomolybdate in both the absence and presence of excess Mg^{++}. In these experiments also the silicomolybdate was effectively in the reduced form, since it was rapidly reduced by the mitochondrial electron transport system in the presence of succinate.

When the mitochondria were allowed to carry out electron transport mediated by silicomolybdate in the absence of metal ions other than Mg^{++} at $1.5 \cdot 10^{-3}$ *M*, their coupling mechanism suffered irreversible damage. The addition of excess Mg^{++} to mitochondria which had been allowed to so respire for 6 min did not subsequently reactivate coupled phosphorylation (Table V). The coupling mechanism sustained a similar fate when conditions of respiratory control (absence of ATP and hexokinase) were imposed on the oxidations (Table V).

TABLE V

INSTABILITY OF PHOSPHORYLATION COUPLED TO SILICOMOLYBDATE OXIDATION IN THE TEMPORARY ABSENCE OF METAL IONS OR ATP

Omitted from complete reaction mixture during first 6 min*	*Oxygen** µatoms 18 min*	*P/O***
—	14.2	0.95
Mg^{++} ($6 \cdot 10^{-3}$ *M*)	12.2	0.06
Hexokinase + ATP	12.6	0.00

* In addition to basic reaction mixture for experiments with ascorbate oxidation as described in text, all flasks contained $6 \cdot 10^{-5}$ *M* silicomolybdate and $7.5 \cdot 10^{-3}$ *M* $MgCl_2$.

** Oxygen consumption and P/O ratio for 18 min reaction period immediately following the addition of the temporarily omitted components.

It was observed that mitochondria have a marked tendency to bind silicomolybdate and that the binding was greatly enhanced in the presence of divalent metal ions. Such information was obtained by the following type of experiment: Silicomolybdate (final concentration of $1.8 \cdot 10^{-4}$ *M*) was added directly to a suspension of mitochondria in 0.3 *M* sucrose containing 8–10 mg protein/ml and metal ions as indicated in Figs. 8 and 9. The mitochondria were then washed by centrifugation (9000 × *g* for 10 min) and resuspension in the original volume of fresh cold sucrose containing metal ions as before but no additional silicomolybdate. Subsequent washings were repeated in the same manner. Figs. 8 and 9 show oxygen uptake by 1 ml (8–10 mg protein) of the washed mitochondria, in a reaction medium containing ascorbate but no additional silicomolybdate, as a function of the number of washings. Repeated washing with the medium which lacked divalent metal ions removed the silicomolybdate and thereby reduced the oxidation rates. In contrast, repeated washing in the presence of divalent metal ions left the oxidation rates essentially unchanged, presumably because the silicomolybdate was strongly bound to the mitochondria under these conditions. Monovalent ions were found to be ineffective in this respect. It should be noted that Co^{++} simulates the action of Mn^{++} in promoting the binding although it does not support phosphorylation coupled to electron transport mediated by silicomolybdate.

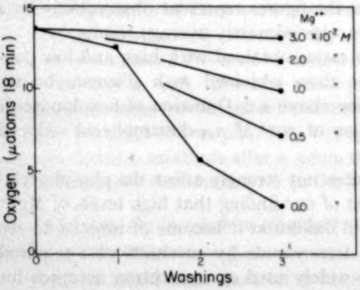

Fig. 8. Effect of Mg^{++} on the binding of silicomolybdate by whole mitochondria. The complete experimental procedure is described in the text.

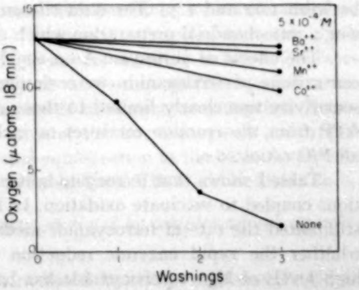

Fig. 9. Effect of various divalent cations on the binding of silicomolybdate by whole mitochondria. Other experimental conditions are identical with those of Fig. 8.

Ferrocyanide

The stimulation of oxygen consumption by addition of ferricyanide to the basic reaction mixture containing ascorbate is shown in Figs. 10 and 11. Phosphorylation was not coupled to these oxidations unless the reaction mixture was further supplemented with Mg^{++} at $7.5 \cdot 10^{-3}$ *M* (Fig. 10) or Ba^{++} (Fig. 11) or Sr^{++} at $2 \cdot 10^{-4}$ *M*. Inactive in this respect were all of the other metal ions tested including Mn^{++}. High levels of Mg^{++} produced greater stimulation of oxidation (Fig. 10) than did the other metal ions.

P/O ratios were somewhat (about 20%) dependent on the concentration of ATP. Maximal ratios were usually obtained when the ATP : ferricyanide ratio was unity in the presence of Mg^{++} and 0.2 in the presence of Ba^{++} or Sr^{++}. These relative concentrations were maintained throughout.

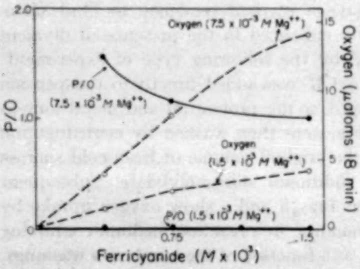

Fig. 10. Effect of $7.5 \cdot 10^{-3}$ *M* Mg^{++} on oxygen consumption and coupled phosphorylation mediated by ferricyanide. Other reaction conditions are identical with those of Fig. 2.

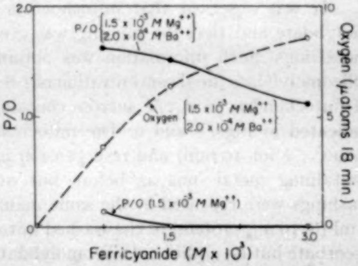

Fig. 11. Effect of $2 \cdot 10^{-4}$ *M* Ba^{++} on oxygen consumption and coupled phosphorylation mediated by ferricyanide. Other reaction conditions are identical with those of Fig. 2.

There was some variation in the observed P/O ratios. For 20 different preparations assayed with $1.5 \cdot 10^{-3}$ *M* ferricyanide and $7.5 \cdot 10^{-3}$ *M* Mg^{++}, the average P/O ratio was 0.94 with a spread between 0.81 and 1.22. With $3.75 \cdot 10^{-4}$ *M* ferricyanide and $7.5 \cdot 10^{-3}$ *M* Mg^{++}, the average P/O ratio for 12 preparations was 1.51 with a spread between 1.82 and 1.34. The data chosen for the figures represent observations on a single mitochondrial preparation which gave approximately average results.

The effects of antimycin A on the P/O ratios obtained with high and low concentrations of ferricyanide were similar to those obtained with silicomolybdate; sensitivity was clearly limited to those ratios above 1.0. Omission of hexokinase or ATP from the reaction mixtures or inclusion of 10^{-4} *M* 2,4-dinitrophenol reduced all P/O ratios to 0.

Table I shows that ferrocyanide itself does not strongly affect the phosphorylations coupled to succinate oxidation. In light of our finding that high levels of Mg^{++} stimulated the rate of ferrocyanide-mediated oxidation it became of interest to see whether the rapid enzymic reduction of ferricyanide by succinate also required high levels of Mg^{++}. Ferricyanide has been widely used as an electron acceptor for oxidative–enzyme systems. Phosphorylation coupled to the reduction of ferricyanide by rat-liver mitochondria in the presence of a variety of substrates of the Krebs cycle has been demonstrated[3, 18, 62] but no unusual requirements for the reaction have yet been reported. PRESSMAN[62] obtained somewhat higher P/O ratios than COPENHAVER AND LARDY[3] with a spectrophotometric assay system but was unable to find a reason for the discrepancy. Table VI shows the unexpected finding that high levels of Mg^{++} had no effect at all on the anaerobic reduction of $6 \cdot 10^{-3}$ *M* ferricyanide by succinate. Identical results were obtained with lower ($1.5 \cdot 10^{-3}$ *M*) concentrations of ferricyanide.

TABLE VI

EFFECT OF Mg^{++} ON ANAEROBIC REDUCTION OF FERRICYANIDE BY SUCCINATE AND OXIDATION OF ASCORBATE-FERROCYANIDE

*Reduction**			*Oxidation***		
Mg^{++} ($M \times 10^3$)	*Ferricyanide (µmoles reduced 5 min)*	*P:2e*	*Mg^{++} ($M \times 10^3$)*	*Oxygen µatoms 18 min*	*P/O*
1.5	14.1	0.43	1.5	3.2	0.0
7.5	12.0	0.45	7.5	12.8	0.92
1.5	10.4	0.31	1.5	4.1	0.0
7.5	11.9	0.30	7.5	13.0	0.89
7.5	0.9***	0.00	7.5	12.5***	0.95

* All flasks contained basic reaction mixture for experiments with succinate oxidation together with 20 µmoles potassium ferricyanide, under anaerobic conditions. Mg^{++} concentration is indicated in Table.

** All flasks contained basic reaction mixture for experiments with ascorbate and $1.5 \cdot 10^{-3}$ *M* ferricyanide.

*** In the presence of 1γ antimycin A.

The binding of ferri- and ferro-cyanide anions by mitochondria is apparently rather weak since their retention could not be demonstrated with the technique of repeated washing, even in the presence of divalent metal ions. Oxygen uptake mediated by residually bound ferricyanide was negligibly small after the very first washing. However, because of this and the fact that ferrocyanide is stable in neutral solution, it was possible to study the characteristics of reactions in which ascorbate was absent and ferrocyanide present in substrate quantities (Table VII). The data clearly confirm that phosphorylation was not efficiently coupled to the oxidation of substrate amounts of ferrocyanide unless special divalent metal ions were added to the system. In these experiments, the rates of oxygen consumption were much less than when ascorbate was used to maintain the ferricyanide totally reduced. The rates decreased slowly with time and consequently about 60-min reaction periods were employed in order to obtain reliable measurements of oxygen consumption. Ferrocyanide (60 µmoles) was placed in the side arms of the reaction vessels and tipped into the reaction mixtures after a 3-min thermal equilibration in the bath. A conservative estimate of maximum error in the P/O ratios of Table VII would be about 10%.

The dependence of the electron transport reactions mediated by complex anions on endogenous cytochrome c

It was found that electron transport mediated by either silicomolybdate or ferricyanide proceeded to molecular oxygen *via* endogenous cytochrome *c*. The mitochondria were depleted of their endogenous cytochrome *c* by suspension in 0.015 *M* KCl and subsequent washing with 0.15 *M* KCl. As judged by the concomitant

TABLE VII

PHOSPHORYLATION COUPLED TO THE OXIDATION OF FERROCYANIDE IN THE ABSENCE OF ASCORBATE

Metal ions *($M \times 10^4$)*	*Oxygen uptake* *(µatoms/60 min)*	*Pi uptake* *(µmoles/60 min)*	*P/O*
Mg^{++} (15)	2.6	0.8	0.31
Mg^{++} (15) ferrocyanide omitted	0.0	0.0	—
Mg^{++} (75)	6.4	7.6	1.10
Mg^{++} (75) ferrocyanide omitted	0.9	0.0	—
Ba^{++} (2)	5.6	6.8	1.22
Sr^{++} (2)	3.0	3.2	1.07
Mn^{++} (2)	3.1	0.8	0.26
Mg^{++} (15)	4.4	0.9	0.20
Mg^{++} (15) ferrocyanide omitted	0.8	0.0	—
Mg^{++} (75)	6.0	6.3	1.06
Mg^{++} (75) ferrocyanide omitted	0.9	0.1	—
Ba^{++} (2)	6.6	8.2	1.24
Sr^{++} (2)	5.0	5.5	1.10
Mn^{++} (2)	4.8	1.4	0.30

All flasks contained basic reaction mixture for experiments with ascorbate except that ascorbate was omitted and $1.8 \cdot 10^{-3}$ *M* $K_4Fe(CN)_6$ was present. Other additions are indicated in Table.

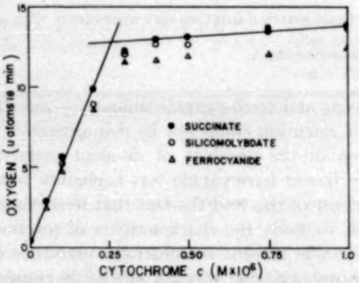

Fig. 12. Restoration by externally-added cytochrome *c* of succinate, ascorbate–silicomolybdate and ascorbate–ferricyanide oxidations in mitochondria depleted of their endogenous cytochrome *c*. All flasks contained the basic reaction mixtures for the respective substrates, $7.5 \cdot 10^{-3}$ *M* Mg^{++}, and where indicated, $6 \cdot 10^{-5}$ *M* silicomolybdate or $1.5 \cdot 10^{-3}$ *M* ferricyanide.

loss of succinate oxidation, this treatment removed more than 95 % of the endogenous cytochrome *c*. Fig. 12 shows that the oxidations of succinate, ascorbate-silicomolybdate and ascorbate-ferricyanide in the extracted preparations were restored with equal efficiency by low concentrations of added cytochrome *c*.

Experiments with DPNH

The literature contains conflicting reports regarding the capacity of intact rat-liver mitochondria to catalyze the direct oxidation of exogenous DPNH. CHANCE AND WILLIAMS[19] and LEHNINGER[20, 21] were unable to observe this reaction. ERNSTER *et al.*[22] obtained slow oxidation with P/O ratios approaching 1.0. MALEY[23] observed good oxidation rates with P/O ratios approaching 3.0. LINNANE AND ZIEGLER[24] have concluded that undamaged beef-heart mitochondria also do not carry out the direct oxidation of DPNH at any significant rate. We did observe the inception of this oxidation, however, following a long and somewhat variable incubation time ranging from 18–60 min. The length of the latent period was considerably prolonged by high levels of Mg^{++} ($7.5 \cdot 10^{-3}$ *M*) or by low levels of Mn^{++}, Sr^{++} or Ba^{++} ($2 \cdot 10^{-4}$ *M*). The oxidation rates progressively increased during the course of the reaction which followed the latent period. Phosphorylation was usually coupled to this delayed oxidation but the maximal P/O ratios (about 1.0) were always associated with the initial (1–2 μatoms) oxygen consumption and extended reaction times invariably led to low ratios. This phenomenon may account for the observations of ERNSTER *et al.*[22] but the high P/O ratios reported by MALEY[23] were never approached in our experiments. Previous workers[20–23] also reported a marked stimulation of DPNH oxdiation upon addition of supplementary cytochrome *c* with final P/O ratios ranging between 0 and 2. ERNSTER *et al.*[22] found that Amytal reduced these P/O ratios close to zero while MALEY[23] reported that antimycin A reduced them to 0.5. Their experiments made in the presence of cytochrome *c* and antimycin A or Amytal show oxidation and phosphorylation rates very similar to ours (Fig. 1, Table VIII) which were generally insensitive to these inhibitors.

TABLE VIII

PHOSPHORYLATION COUPLED TO DPNH OXIDATION MEDIATED BY VARIOUS ELECTRON CARRIERS

Catalyst	*Mg++ (M × 10³)*	*Additions*	*Oxygen (μatoms 18 min)*		*P/O*	
			Expt. 1	*Expt. 2*	*Expt. 1*	*Expt. 2*
—	1.5	—	0.95	1.1	—	—
—	7.5	—	0.0	0.9	—	—
Silicomolybdate ($1.5 \cdot 10^{-5}$ *M*)	1.5	—	12.7	10.5	1.03	1.00
Silicomolybdate ($1.5 \cdot 10^{-5}$ *M*)	7.5	—	12.5	10.4	1.01	1.05
Silicomolybdate ($1.5 \cdot 10^{-5}$ *M*)	1.5	ascorbate ($7.5 \cdot 10^{-3}$ *M*)	12.1	12.5	1.10	1.09
Silicomolybdate ($1.5 \cdot 10^{-3}$ *M*)	1.5	antimycin (1 μg)	12.0	10.0	1.05	0.95
Silicomolybdate ($6 \cdot 10^{-5}$ *M*)	1.5	—	12.0	9.8	1.01	1.00
Silicomolybdate ($6 \cdot 10^{-5}$ *M*)	7.5	—	9.7	8.5	0.78	0.84
Ferricyanide ($3 \cdot 10^{-3}$ *M*)	1.5	—	0.90	1.2	—	—
Ferricyanide ($3 \cdot 10^{-3}$ *M*)	7.5	—	12.1	11.9	1.14	1.08
Ferricyanide ($3 \cdot 10^{-3}$ *M*)	1.5	Ba^{++} ($2 \cdot 10^{-4}$ *M*)	1.0	1.9	—	—
Cytochrome *c* ($1.5 \cdot 10^{-5}$ *M*)	7.5	—	10.6	11.5	0.35	0.41
Cu^{++} ($1.5 \cdot 10^{-5}$ *M*)	7.5	—	13.5	12.6	0.10	0.05
Menadione ($1.5 \cdot 10^{-5}$ *M*)	7.5	—	11.4	9.1	0.20	0.43

All flasks contained the basic reaction mixture for experiments with DPNH as indicated in the text. Other additions are indicated in Table.

We have found that a variety of substances could mediate the oxidation of exogenous DPNH by rat-liver mitochondria. Figs. 13 and 14 compare the capacity of silicomolybdate, ferrocyanide, menadione, Cu^{++} and cytochrome *c* to act in this fashion. Fig. 14 shows that the electron-transport reactions mediated with ferrocyanide could only proceed in the presence of high levels of Mg^{++} ($7.5 \cdot 10^{-3}$ *M*). No other metal ions could replace Mg^{++} in this respect. The electron-transport reactions mediated by each of these five carriers were totally blocked by 10^{-3} *M* KCN but insensitive to antimycin A and $1.8 \cdot 10^{3}$ *M* Amytal. Phosphorylation was efficiently coupled only to those oxidations mediated by silicomolybdate or ferricyanide (Table VIII). The data of Table VIII clearly demonstrate that the P/O ratios obtained with DPNH-silicomolybdate are maximal even at levels of Mg^{++} ($1.5 \cdot 10^{-3}$ *M*) for which the corresponding ratios obtained with ascorbate-silicomolybdate were close to zero. Fig. 13 also shows that silicomolybdate and cytochrome *c* were much less effective in mediating electron-transport reactions when maintained reduced with ascorbate rather than with DPNH. The concentration of ascorbate ($1.5 \cdot 10^{-2}$ *M*) used in these experiments produced oxidation rates close to those obtained at infinite ascorbate concentration. Addition of ascorbate to the DPNH system did not inhibit oxidation or coupled phosphorylation.

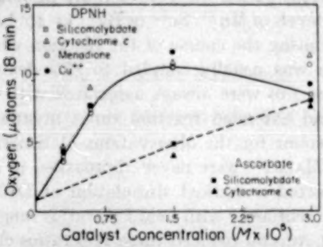

Fig. 13. Electron transport mediated by various electron carriers with $6 \cdot 10^{-3}$ *M* DPNH and $1.5 \cdot 10^{-2}$ ascorbate. All flasks contained the basic reaction mixtures for the respective substrates as described in the text, $7.5 \cdot 10^{-3}$ Mg^{++} and the concentrations of the electron carriers indicated in the figure. The concentrations of ATP used with DPNH were identical with those for ascorbate–silicomolybdate oxidations.

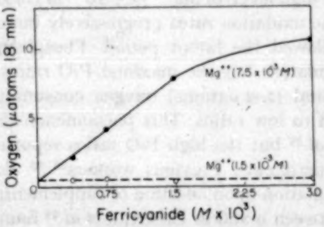

Fig. 14. Electron transport mediated by ferricyanide in the presence of DPNH. Data are shown for two concentrations of Mg^{++}. Other experimental conditions are identical with those of Fig. 13 that the concentrations of ATP were identical with those for ascorbate–ferricyanide oxidations.

The non-enzymic reduction of cytochrome *c*, silicomolybdate and ferricyanide by DPNH proceeded at a negligibly slow rate. Upon addition of mitochondria, under either aerobic or anaerobic conditions, each of these electron carriers was rapidly reduced, as shown by instantaneous development of the characteristic color of the reduced compound. The rate of reduction of ferricyanide by exogenous DPNH in the presence of the mitochondria was found to be totally independent of Mg^{++} concentration.

The reductase activity responsible for the observations reported in this section could presumably originate with the microsomes which invariably contaminate mitochondrial preparations. However, extensive washing of the mitochondria with 0.3 *M* sucrose failed to reduce this activity. The possibility must also be considered that an "external" DPNH-coupled reductase, capable of rapidly reducing a number of compounds, resides on the outer surface of the mitochondria. The existence of such an enzyme was first suggested by LEHNINGER[20].

RAW *et al.*[25] have recently published an account of the purification and properties of a DPNH-cytochrome *c* reductase extracted from the mitochondria of pig, guinea-pig, and rabbit livers. These workers were able to resolve the enzyme into a "556" cytochrome and a flavoprotein. Their purified flavoprotein was able to reduce directly ferricyanide and cytochrome 556 but not cytochrome *c*. Presumably menadione and cytochrome *c* were reduced via the cytochrome 556. Functionally related reductases[26, 27] have also been obtained from beef- and pig-heart mitochondria.

The possible role of an "external" DPNH-cytochrome *c* reductase in the interaction of both DPNH and the complex anions with the terminal electron-transport system of the mitochondria was further investigated by controlled removal of the antimycin-insensitive reductase activity from the mitochondria. LEHNINGER[21] and PRESSMAN AND DE DUVE[57] had previously observed that mitochondria exposed to hypotonic media oxidized DPNH much more rapidly than mitochondria maintained in an isotonic environment. The oxidations induced by the hypotonic exposure were considerably insensitive to antimycin A. In our experiments rat-liver mitochondria were exposed to distilled water containing 0.002 *M* histidine, 0.001 *M* phosphate and 0.002 *M* Tris, adjusted to pH 6.0. A sufficient volume of this solution was used such that solubilized proteins did not alter the initial pH (6.0). The pH of the mitochondrial suspension was then gradually increased by slow addition of dilute KOH. Aliquots were taken out at various points and centrifuged at 100,000 × *g* for 10 min. The supernatant was stored and the residue resuspended in unbuffered 0.3 *M* sucrose. Table IX shows the oxidation rates and P/O ratios effected by these residues with succinate, β-hydroxybutyrate and DPNH. Table IX also shows the relative DPNH-cytochrome *c* reductase activity in the supernatants. The data clearly show an inverse relationship between this activity and the capacity of the residue to oxidize rapidly the exogenous DPNH via an antimycin-insensitive pathway. The mitochondria exposed to the hypotonic medium at pH 6.0 showed the greatest increase in DPNH

TABLE IX

OXIDATIONS AND COUPLED PHOSPHORYLATION IN MITOCHONDRIA EXPOSED TO HYPOTONIC MEDIA OF INCREASING pH

pH of hypotonic medium	*Succinate*		*β-Hydroxybutyrate*		*DPNH*		*DPNH-cytochrome c reductase activity*** of supernatant*
	Oxygen (μatoms/18 min)	*P/O*	*Oxygen* (μatoms/18 min)*	*P/O*	*Oxygen (μatoms/18 min)*	*P/O*	
Control (0.3 *M* sucrose)	13.1	1.69	5.8	2.65	0.9	—	
6.0	12.1	0.95	5.1	1.69	14.2	0.2	0.04
6.0	0.0**	—	0.0**	—	10.9**	0.0	
6.5	13.5	0.91	4.9	1.65	9.3	0.51	0.13
7.0	13.2	0.90	4.8	1.61	5.9	1.08	0.18
7.5	15.1	0.81	5.2	1.49	4.3	1.39	0.26
7.5	0.0**	—	0.0**	—	0.5**	—	
8.0	20.1	0.61	6.1	1.11	4.4	1.08	0.25
8.5	16.3	0.20	5.9	0.39	3.9	0.30	0.29
9.0	14.8	0.11	6.9	0.10	4.5	0.0	0.35

All flasks contained basic reaction mixture for experiments with succinate oxidation and the residue derived from 8 mg protein of whole mitochondria. Where indicated $7.5 \cdot 10^{-3}$ *M* β-hydroxybutyrate or $6 \cdot 10^{-3}$ *M* DPNH replaced succinate.

* Plus $3 \cdot 10^{-4}$ *M* DPN.

** Plus 1 γ antimycin A.

*** ΔA_{550} mμ/min/mg protein in 0.01 potassium phosphate, pH 7.0.

oxidation activity, over 2/3 of which was insensitive to antimycin A. The data of Table IX are consistent with the idea that when the pH of the hypotonic medium is raised, the antimycin-insensitive reductase is obtained in the centrifuged supernatant while the sensitive pathway remains in the residue. The residual oxidation appears coupled to phosphorylation.

Attempts were made to purify the reductase activity released into the soluble phase between pH 6.0 and 8.0 by protamine and ammonium sulfate precipitation. A 10- to 20-fold purification was usually achieved in this way. Further purification of the reductase as a soluble enzyme proved to be extraordinarily difficult. Additional ammonium sulfate fractionation produced a precipitate which could not be redissolved below pH 10. The same phenomenon was observed with isoelectric precipitation at pH 5.1. Attempts to purify the enzyme by adsorption on cellulose anion-exchange resins were generally unsuccessful; the activity collected in a narrow, intensely yellow band at the top of the column which subsequently could not be eluted with phosphate or Tris at various pH values and concentrations. The enzyme displayed strong reductase activity in either the adsorbed, precipitated or soluble states. The absorption spectrum of preparations purified more than 10 times always showed a characteristic cytochrome "*b*" absorption peak at 556 mμ upon reduction with DPNH. A broad flavin absorption at 450–460 mμ and a Soret band at 415 mμ were also evident Further work on the purification and properties of this reductase is in progress since the experiments described above are not sufficient reason to exclude the possibility that the reductase activity is of microsomal origin.

DISCUSSION

The data presented in this paper demonstrate that phosphorylation is efficiently coupled to electron transport mediated by high potential electron carriers only if special factors, not required to obtain maximal P/O ratios with succinate oxidation, are present in the reaction medium. When mitochondria are depleted of their endogenous cytochrome *c*, oxidation of both succinate and the reduced complex anions is dependent on addition of external cytochrome *c* (Fig. 12), suggesting that the pathway of terminal electron transport is likely to be the same in both systems. Previous workers[1–8] who studied electron transport reactions mediated by cytochrome *c* overlooked the general scope of this phenomenon, perhaps because they routinely included fluoride or EDTA in their experimental media. NIELSEN AND LEHNINGER[6] attributed the EDTA requirement for phosphorylation coupled to the oxidation of ferrocytochrome *c* to contamination of their mitochondria by heavy metal ions. Although this explanation can not be excluded, the similar activity of a variety of other ions in the several related reaction systems described in this paper now seems to indicate the existence of a more basic requirement for factors which can stabilize some critical feature of the mitochondrial structure characteristically unstable to electron transport mediated by high potential electron carriers.

There is considerable evidence in the literature[28–42] supporting the concept that structural stabilization is an essential factor for optimal phosphorylation in intact rat-liver mitochondria. BALTSCHEFFSKY[41] has shown that the stability of respiratory control of succinate oxidation by fresh mitochondria in a magnesium-free medium is markedly enhanced under mildly alkaline (pH 8.0) reaction conditions.

This phenomenon is undoubtedly related to our observation (Table I) that a mildly alkaline reaction medium (pH 7.6) containing low ($1.5 \cdot 10^{-3}$ *M*) levels of Mg^{++} is as optimal an environment for fresh mitochondria oxidizing succinate as a neutral medium containing high ($7.5 \cdot 10^{-3}$ *M*) levels of Mg^{++}. As the mitochondrial structure becomes progressively weakened by aging, however, excess Mg^{++} ions apparently can no longer provide the required degree of stabilization during electron transport. Under these conditions BSA[42], Mn^{++} and OH^- (Table III) are partially effective in maintaining phosphorylation coupled to succinate oxidation. Apparently the structure of even freshly prepared mitochondria is excessively strained during electron transport mediated by high potential electron carriers since, as shown in this paper, phosphorylation coupled to such electron transport depends on the presence of factors which are not required for phosphorylation coupled to the oxidation of succinate which is a natural source of electrons for reducing the endogenous cytochromes. This phenomenon does not appear to be due to an inhibitory action of ascorbate, cytochrome *c*, silicomolybdate or ferrocyanide since these compounds do not seriously affect phosphorylation coupled to succinate oxidation (Table I), even though mitochondria bind exogenous cytochrome *c*[43,63] and silicomolybdate (Figs. 8 and 9).

BRENNER-HOLZACH AND RAAFLAUB[33], RAAFLAUB[31,32], and ERNSTER AND LÖW[28] have advanced the concept that some structural feature of mitochondria, critically related to phosphorylative capacity, is maintained by endogenous ATP and magnesium. BALTSCHEFFSKY[41] postulated the existence of some mitochondrial component with a p*K* around 7.5 whose ionization greatly influences the stability of mitochondrial structure and function. SIEKEVITZ AND POTTER[39] offered evidence that fluoride forms a complex with bound Mg^{++} and thereby prevents its discharge from the mitochondria. It is conceivable that hydroxyl, EDTA, phosphotungstate and divalent metal ions can also form complexes with endogenous components and thereby stabilize the mitochondrial structure under the present conditions of electron transport.

The data of Fig. 13 and Table VIII show that electron transport from substrate to oxygen is more efficiently mediated through silicomolybdate and cytochrome *c* when the substrate is exogenous DPNH rather than ascorbate, and that maximal rates of oxidation and phosphorylation no longer require addition of special factors. Our data suggest that with DPNH these electron carriers are first reduced by a non-phosphorylative DPNH-coupled reductase which is presumably localized on the outer surface of a mitochondrion and subsequently donate their electrons to the internal cytochromes. LEHNINGER[30] has suggested that both of these steps proceed *via* an external pathway, presumably because he found oxygen consumption initiated under these conditions to be essentially non-phosphorylative. Our observations, however, with silicomolybdate and ferrocyanide (Table VIII) tend to support the suggestion that some segment of the internal phosphorylative electron transport pathway is included in the oxidation reactions.

The higher oxidation rates obtained with DPNH compared to those with ascorbate imply that the former substrate is able to maintain a higher local concentration of reduced carrier in the region of the internal cytochromes. This could be due to an increase in the permeability of the mitochondrial surface induced by the DPNH or a capacity of DPNH to effect reduction of the carrier molecules behind the permeability barrier. SLATER[17] previously discussed this situation with respect to the capacity of ascorbate to reduce effectively the exogenous cytochrome *c*. Since the rapid interaction of ferrocyanide with the internal cytochromes requires high levels of Mg^{++} whether maintained reduced with ascorbate or DPNH (Figs. 10 and 14), it can be further suggested that Mg^{++} also has the capacity to effect changes in the mitochondrial surface. The anomalous behavior of ferrocyanide in this respect may be related to the fact that mitochondria generally bind it much less strongly than cytochrome *c* or silicomolybdate.

The mode of action of divalent metal ions in the electron transport reactions mediated by complex anions may be related to their capacity to enhance the binding of silicomolybdate by the mitochondria. The data are inadequate to decide whether the metal ions form a direct bridge between silicomolybdate and mitochondrial protein or induce structural or electrostatic modifications of the protein which subsequently favor enhanced binding and increased stability. The former explanation is analogous to the postulated[28,31] binding of ATP to mitochondria through the intermediary of Mg^{++} or Mn^{++}. Stabilization of phosphorylation coupled to succinate oxidation in Ca^{++}-inactivated mitochondria by Mg^{++}, Mn^{++}, Co^{++} and Ni^{++} has been reported by ERNSTER AND LÖW[28]. Likewise, stabilization of phosphorylation coupled to DPNH oxidation in sonicates of mitochondria by Mn^{++}, Co^{++} and Fe^{++} has been observed by BRONK AND KIELLEY[44]. It would appear as if stabilization of phosphorylation by divalent metal ions may be quite a general effect. In contrast, however, COOPER AND LEHNINGER[45] observed an uncoupling action of exogenous Mg^{++} with digitonin extracts of mitochondria. These preparations were reported to contain considerable bound Mg^{++}. The failure of Mn^{++} to support phosphorylation coupled to the oxidation of ascorbate mediated by ferricyanide but not that mediated by silicomolybdate may be related to the tendency of Mn^{++} to form insoluble salts with ferrocyanide. The inability of Co^{++} and Ni^{++} to elicit phosphorylation with either complex anion may be due to a similar phenomenon. It has previously been shown[46,47] that Mg^{++} and Mn^{++} antagonize the uncoupling of oxidative phosphorylation by iodo-thyronines—a close parallel to their effect on the phosphorylations coupled to silicomolybdate oxidation. Since it now appears[48–50] that thyroxine tends to labilize mitochondrial structure, a somewhat similar role for silicomolybdate may be inferred from this body of information. The action of silicomolybdate, however, seems additionally related to its capacity to mediate electron transport since phosphorylation coupled to succinate oxidation is not significantly affected by the presence of silicomolybdate (Table I), suggesting that the act of binding *per se* does not elicit loss of phosphorylative capacity.

In contrast to our finding with whole mitochondria that P/O ratios associated with the oxidations mediated by cytochrome *c* are maximal near pH 8, where oxidation rates are also large (Fig. 1), COOPER AND LEHNINGER[51] observed that with digitonin extracts derived from whole mitochondria, corresponding P/O ratios were maximal at pH 5.5, where oxidation rates were minimal. HÜLSMANN AND SLATER[8] reported a maximum near pH 6 in guinea pig heart mitochondria, but their experiments were made in the presence of $6 \cdot 10^{-4}$ *M* EDTA which we found (Table II) would independently enhance the P/O ratio. A comparison of these data, however, may not be justified since the pH curves probably represent different phenomena. The stimulation of cytochrome *c*-mediated oxidation by hydroxyl ions (Fig. 1) may be related to the induction of mitochondrial swelling, known[52] to occur at pH 8, with a concomitant increase in permeability. It is not clear why this would also lead to higher

P/O ratios. A similar change in permeability was shown by LEHNINGER *et al.*[5] to accompany exposure of mitochondria to hypotonic media.

The redox potential of the silicomolybdate couple, extrapolated to pH 7.0 from measurements made between pH 2.5–4.5 with a Beckman platinum electrode, was found to be $E'_0 = +0.45$ V. STRICKLAND[53] estimated the value to be near +0.56 V. The potential level of the anion bound to mitochondrial protein may vary somewhat from this value. The redox potential of the ferri- ferro-cyanide couple[54] (E'_0) corrected for potassium-ion effects, is +0.44 V. Although the potentials are comparable, the concentration of ferrocyanide required to catalyze maximal rates of oxygen consumption with the mitochondria in the presence of ascorbate was found to be 25–50 times greater than that of silico-12 molybdate. Assuming that the electron-transport processes can be initiated only by those complex anions which enter or become bound to some special region of the mitochondria, this phenomenon can be ascribed to a difference in binding or rate of penetration of the two complex anions. This is supported by the data in Figs. 8 and 9. Such differences could be related to the nature of the charged groups on the surfaces of these large anions.

The data of Table VII clearly demonstrate that the oxidation and coupled phosphorylation observed with ferrocyanide is independent of ascorbate. It seems reasonable to assign a similar passive role to ascorbate in the reactions mediated by other high-potential donors like silicomolybdate and cytochrome *c*. The P/O ratios reported in Table VII indicate a remarkably efficient coupling between phosphorylation and electron-transport reactions initiated by ferrocyanide. We have demonstrated (Fig. 12) that electron transport mediated by ferrocyanide can only proceed to molecular oxygen *via* endogenous cytochrome *c*. In order to get an estimate of the potential level of the mitochondrial component to which ferrocyanide in the absence of ascorbate donates electrons, a kinetic analysis was made of the time course of oxygen consumption with ferrocyanide as substrate, using an appropriate simple reaction scheme*. An E'_0 value close to that of cytochrome *c* was obtained, suggesting that phosphorylation coupled to electron transport initiated by ferrocyanide in the absence of ascorbate is primarily that associated with the oxidation of an endogenous component near the potential level of cytochrome *c*, which has been non-enzymically reduced *in situ* by ferrocyanide. In the presence of ascorbate, mitochondrial components with redox potentials considerably below that of cytochrome *c* could be reduced by ferrocyanide. The P/O ratios greater than 1.0 and approaching 2.0, obtained with ascorbate and the complex anions clearly indicate that the complex anions can feed electrons into the respiratory chain at two different levels. The characteristic increase in P/O ratios with decreasing concentrations of electron carrier were observed in all of the electron transport reactions studied.

LEHNINGER[20] has previously suggested that the surface of intact mitochondria is impermeable to external DPNH but contains an enzyme which can couple its oxidation to the reduction of external cytochrome *c*. He also suggested that exposure of the mitochondria to hypotonic media removed this permeability constraint and thereby created a pathway for the direct oxidation of external DPNH by the internal cytochrome chain. The following more complicated picture of DPNH oxidation seems to be consistent with our data:

(1) The outer surface of intact mitochondria contains an enzyme or enzyme

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* Unpublished work of M. Gellert and E. Jacobs.

complex which can couple the oxidation of external DPNH to the rapid reduction of external cytochrome *c*, silicomolybdate, menadione and ferrocyanide, but not to the reduction of the internal electron transport chain which itself appears to reside on a well-defined structure[60]. The first three of the above carriers can freely donate electrons to the internal electron transport chain in the presence of external DPNH alone while ferrocyanide additionally requires high levels of Mg^{++} in order to do this. In our experiments the pathway between external DPNH and oxygen which was completed by each of the above electron carriers was totally insensitive to antimycin A and Amytal. These results are consistent with previous studies using cytochrome *c* as the mediator[20, 22, 23]. Menadione oxidation in heart preparations, on the other hand, have been reported to be sensitive to antimycin A[55]. The significance of this difference is not discernable at present. In our experiments with external DPNH oxidation, the antimycin-insensitive electron transport to oxygen mediated by silicomolybdate and ferricyanide always gave P/O ratios of 1.0 whereas that catalyzed by external cytochrome *c* and menadione always resulted in P/O ratios less than 0.5.

(2) Exposure of the mitochondria to hypotonic media at pH 6 induces swelling and structural damage, thereby permitting direct interaction between the external reductase and the internal electron-transport chain. Since extraction of endogenous cytochrome *c* inactivates this newly created pathway and since the reaction is not inhibited by antimycin A, the point of interaction would appear to be around cytochrome *c*. Under these conditions we have observed that the rate of oxidation of external DPNH in the absence of supplementary electron carriers is maximal and that very little esterification of inorganic phosphate accompanies the oxidation. We have also always observed that the P/O ratios accompanying oxidation of substrates of the Krebs cycle drop by about 1.0 when this situation obtains (Table IX). Assuming that the cytochrome oxidase chain is identical for all of the above-mentioned oxidations we are led to postulate that hypotonic exposure rapidly inactivates the coupled phosphorylation in this segment of the respiratory chain. PRESSMAN[62] had previously suggested that some loss of phosphorylative capacity accompanies exposure of mitochondria to hypotonic media. Whether or not the observed inactivation can be attributed to a collapse of two juxtaposed surfaces is at present being investigated.

(3) The external reductase is stripped off from the mitochondrion as the pH of the hypotonic medium is raised from 6.0 to 8.0, thereby destroying the artificially-created shunt pathway between DPNH and oxygen. After the external reductase has been removed in this manner, oxidation of added DPNH becomes slower and more commensurate with the rates of oxidation of endogenous DPN-linked substrates, antimycin-sensitive and efficiently coupled to phosphorylation (Table IX). Presumably the oxidation of external DPNH is now proceeding by the same pathway as that utilized by the DPN-linked substrates. Previous investigations[56–58] of DPNH oxidation in relation to DPNH-cytochrome *c* reductase activity and its localization following exposure of mitochondria to distilled water have not described the above mentioned effect of pH, possibly because the pH of unbuffered distilled water found in many laboratories lies between 5.5–6.0. We have consistently observed that whenever mitochondria were subjected to any treatment which enables them to oxidize rapidly external DPNH in the absence of supplementary electron carriers, a concomitant decrease of about 1.0 appears in the P/O ratios for all substrate oxidations. LINNANE AND ZIEGLER[24] have suggested that DPNH oxidation can give P/O

ratios of only 2 and that the third phosphorylation step may lie between substrate and DPN. It would appear as if these authors have not considered the possibility that the loss of a phosphorylation in the cytochrome oxidase segment of the respiratory chain may be induced by treatments which degrade the mitochondrial structure. Cooper and Lehninger[45] have reported that particles which can directly oxidize DPNH obtained from digitonin extracts of mitochondria show P/O ratios approaching 3.0 for β-hydroxybutyrate oxidation. However, more recent work by Devlin and Lehninger[59] indicates that these P/O ratios approach 2.0 rather than 3.0 as an upper limit. Table VIII shows that the corresponding P/O ratios obtained with mitochondria disrupted by exposure to hypotonic media below pH 7.8 also approaches 2.0. Siekevitz and Watson[60] have shown that low concentrations of deoxycholate induce mitochondrial swelling in a fashion similar to that effected by distilled water. It would appear, therefore, that structural changes produced in mitochondria by the usual type of mild physical or chemical treatment, including exposure to digitonin, generally lead to a lowering of P/O ratios by 1.0.

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FURTHER OBSERVATIONS ON THE ADENOSINE PHOSPHATASES OF COBRA VENOM

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SUMMARY

Dried cobra venom has been dialysed under various conditions. The effect on the $\alpha\beta$-ATP-ase, 5′-nucleotidase and DPN-*pyro*phosphatase, also that of chelating agents and of certain metal ions, is reported. The effect of specific antiserum has been investigated. The venom hydrolyses inosine triphosphate and coenzyme A in a manner analogous to the breakdown of ATP and DPN respectively. Flavine mononucleotide, ribose-5-phosphate and nicotinamide mononucleotide are hydrolysed at only 0.5, 1 and 2 % of the rate of adenosine monophosphate hydrolysis. Thiamin *pyro*phosphate is not hydrolysed.

INTRODUCTION

In a previous publication[1] an enzyme system of cobra venom was described which breaks down ATP by means of $\alpha\beta$-ATP-ase and 5′-nucleotidase[2] liberating *pyro*-P, *ortho*-P and adenosine. This system occurs in other snake venoms[3,4] and in semen[5], from which sources both enzymes have been studied in the partially purified state. The dried venom available to the author, however, contained practically no active non-specific phosphatase or $\beta\gamma$-ATP-ase, thus facilitating a direct study of the venom enzymes mentioned above.

MATERIALS AND METHODS

ATP, ADP and AMP were those described in an earlier publication[1]. ITP and IMP were prepared by Mr. D. H. WILLIAMSON according to the method of KLEINZELLER[6]. The IMP was almost 100 % pure. The ITP analysed as follows: ITP, 48.4: IDP, 42.3: IMP, 4.7: *ortho*-P, 3.7: *pyro*-P, 1.0 %. DPN was obtained from yeast by the method of LEPAGE[7]. (See Table VII, col. 2 for analysis.) FMN, reputed to be 90 % pure, was kindly supplied by the Sigma Chemical Company. Chromatography showed two fluorescent spots with R_F values of 0.095 and 0.129. The lower spot contained 14 % of the phosphorus and 5.5 % of the fluorescence. TPP solution (20 m*M*) was kindly supplied by Dr. W. BARTLEY. It contained a trace of *ortho*-P. R-5-P was prepared by Mr. D. H. WILLIAMSON by acid hydrolysis of NMN and isolated as the Ba salt. Aqueous solutions of the Na salt were prepared as required. Coenzyme A was a 50 % pure sample made by the method of KAPLAN AND LIPMANN[8] and given by Mrs. M. HOKIN. Run in *n*-butanol–acetic acid–water mixture, it showed under u.v. light one spot R_F 0.013 and one in the AMP area, both containing P. All stock chemicals were of A.R. purity.

Cobra (Naia Naia) *venom* was the dried material described previously[1]. Stock solutions containing 5.4 mg/ml were made up in water or in 0.08 *M* veronal buffer, pH 8.4. Between pH 7.0 and 9.0 in veronal buffer the venom activity towards mono- and diphenylphosphates was about 1.0 and 0.2 % respectively of that towards AMP and DPN. The natural, non-specific phosphatases[9] were presumably inactivated during drying and storage of the venom. No $\beta\gamma$-ATP-ase was present. No phosphates or u.v. light-absorbing materials were detected in the venom (*cf.* DOERY[10]). Venom in veronal buffer, pH 8.4, was used in some experiments after a preliminary dialysis at 20°, overnight against similar buffer. This is termed "buffer-dialysed" venom solution. Venom dialysed otherwise is described where necessary. All proportions by weight, and percentages refer to the weight of original dried (not dialysed) venom.

Antiserum was the dried material described previously[1]. It was weighed as required and dissolved in the buffer solution. No contaminating phosphates or u.v. light-absorbing materials were detected.

All solutions were stored at —20° without deterioration.

The following abbreviations are used in the text: ATP, ADP, AMP, adenosine-5′-tri-, di-, and monophosphate; ITP, IDP, IMP, inosine-5′-tri-, di-, and monophosphate; DPN, diphosphopyridine nucleotide; TPP, thiamin *pyro*phosphate; *ortho*-P and *pyro*-P, inorganic *ortho*- and *pyro*-phosphate; FMN, flavine mononucleotide; NMN, nicotinamide mononucleotide; R-5-P, ribose-5-phosphate; EDTA, ethylenediaminetetraacetic acid; tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid.

Estimation of Mg^{++} *and* Zn^{++}: The colorimetric method of GARNER[11] was used to determine Mg^{++} in the venom and in a TCA extract, both after incineration in a platinum crucible at dull red heat. Potassium cyanide (5 %, 0.1 ml per 0.5 ml of solution) was used to overcome the inhibitory effect of Zn^{++} on colour-formation. The values obtained were about 2.0 mg and 1.3 mg/g dried venom respectively. Zn^{++} was determined with dithizone[12], 2.2 mg/g dried venom was found. These values correspond to a concentration in the natural venom of 29 mM Mg^{++} and 12 mM Zn^{++}, assuming a 33 % solid content and a specific gravity of 1.058 for the natural venom[13]. The dried venom left 2.14 % ash, most of which was removed on water dialysis. It contained no detectable manganese or cobalt.

Nicotinamide determination: AMP and NMN, the initial breakdown products of DPN, run together when paper-chromatographed in *n*-butanol–acetic acid–water (see Table I). For the determination of the NMN in the eluted spot the nicotinic acid produced by alkaline hydrolysis was estimated by the method of BANDIER AND HALD[14] with certain modifications introduced to avoid the formation of dark colours: viz. addition of extra CNBr after the heating process, to stabilise the metol; preparation of CNBr, ice cold, immediately before use; its addition to the solution before heating; use of metol within 1 h and decolorisation with charcoal if necessary. A reproducible straight-line graph was obtained giving 3 % accuracy. The necessary chromatogram areas were eluted with water. AMP did not affect the result.

TABLE I

R_F VALUES OF SOME SUBSTANCES IN *n*-BUTANOL–ACETIC ACID–WATER

The substances were run in the solvent upper layer by descending chromatography at about 20° for varying periods on No. 4 Whatman paper. The values were obtained with mixtures 2 to 7 days old.

Substance	R_F	Substance	R_F
DPN	0.013	AMP	0.12
ITP	0.017	R-5-P	0.12
ATP	0.020	*Ortho*-P	0.22
ADP	0.033	Guanine	0.23
TPP	0.035	Inosine	0.29
IDP	0.039	Hypoxanthine	0.37
Pyro-P	0.062	Adenosine	0.42
FMN	0.095	Adenine	0.50
IMP	0.10	Phenylphosphate	0.73
NMN	0.11	Diphenylphosphate	0.93

Incubation and sampling were carried out essentially as described previously[1], with modifications where necessary. Venom concentrations of 0.11–0.14 % were normally obtained giving about 85–111 μM magnesium and 35–45 μM zinc. Natural cobra venom is about 300 times more concentrated than the final incubation mixtures used in these experiments (see[13]).

Chromatographic separation and analysis: Separation of most substances was effected as described previously[1]. When only the *pyro*-P was required from ATP or ITP breakdown the formic acid:isopropyl ether solvent mixture of HANES AND ISHERWOOD[15] was used. Mixtures of adenosine phosphates and DPN were resolved by the method of KREBS AND HEMS[16]. No. 4 Whatman paper was used throughout.

Enzymic "activities" are compared using an arbitrary unit, being μmoles/ml of substrate hydrolysed in 30 min at 30° by a 0.1 % cobra venom solution, or by the enzyme equivalent to this amount of venom. Approximate optimal substrate concentrations were determined for ATP, ADP, AMP and DPN. They are, respectively 1.4–1.7; 12–13; 2.6–3.0; 12–20. The corresponding enzyme activities are 1.4–1.5; approx. 1.5; 3.4–3.6; 8.9–11. Apparent Michaelis constants are 0.4–0.5; 3.0; 0.11; 5.0.

RESULTS

Hydrolysis of adenosine phosphates

Effect of pH change on 5′-nucleotidase and ATP-ase: The optimum pH of the venom 5′-nucleotidase was 6.8 to 7.0 (phosphate buffer). The "activity" in veronal buffer was similar at this pH but decreased more rapidly on the alkaline side (see Fig. 1). This may represent an inhibition by the barbiturate ion, the concentration of which is very small at pH 7.0 (where the free acid crystallises readily). In tris buffer activities were increased at all pH values. The ATP-ase optimum was pH 8.6–8.8 (veronal buffer) (*cf.* HEPPEL AND HILMOE[4]). At pH 8.2 tris buffer effected 163 % enhancement. At pH 8.5 borate buffer (0.1 *M*) inhibited 5′-nucleotidase 40 % and ATP-ase 15 % compared with veronal buffer. Similar borate inhibition and/or tris activation has been reported for these enzymes from semen[3,4] and retina[17]. *Pyro*-P (30 m*M*) inhibited 5′-nucleotidase 67 % and ATP-ase 38 % in veronal buffer, pH 8.4. One tenth of this *pyro*-P concentration had no significant effect upon either enzyme.

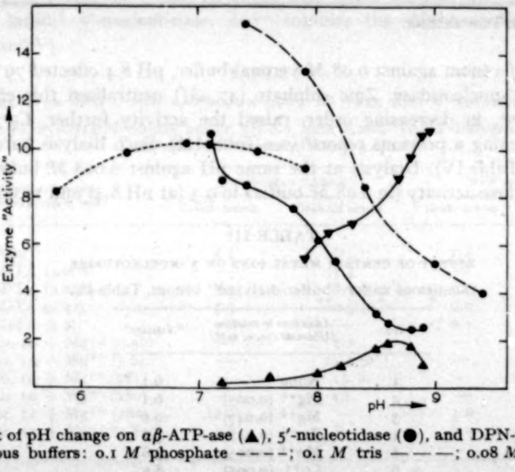

Fig. 1. Effect of pH change on $\alpha\beta$-ATP-ase (▲), 5′-nucleotidase (●), and DPN-pyrophosphatase (▼) in various buffers: 0.1 *M* phosphate – – – –; 0.1 *M* tris — — —; 0.08 *M* veronal ———.

The pH stability (7 days at room temperature) of 5′-nucleotidase was examined at the following pH values: 2.9, 4.3, 5.8 (acetate buffer); 7.0, 8.4 (veronal buffer). The activities (determined in veronal buffer pH 7.0) were respectively 0.03, 4.7, 17.2, 11.9, 15.6.

Dialysis of cobra venom and addition of metal ions: $ZnSO_4$ and $MgCl_2$ added to the crude venom solution up to 1.15 mM had no effect on either enzyme.

Dialysis of the venom at 20° against water effected a reduction of both enzymic activities and ultimately removed most of the Mg^{++} and Zn^{++}. Table II shows these results, and the effect of Mg^{++} and Ca^{++} on the dialysed enzymes. During the dialysis a precipitate formed, which contained 25 % of the 5′-nucleotidase activity. Dialysis against distilled water at 0° for 14 days reduced the 5′-nucleotidase activity (veronal buffer, pH 8.4) to 70 % of its former value while dialysis against 0.9 % KCl at 0° reduced it to 96 %.

TABLE II

EFFECT OF WATER-DIALYSIS ON ATPASE AND 5′-NUCLEOTIDASE

Venom (0.54 %) dialysed against distilled water at 20°, then used at 0.14 %. Incubation at 30° in 0.025 M veronal buffer, pH 8.4.

Time of dialysis (days)	*Total zinc/g venom (μg)*	*Mg^{++}*/g venom (μg)*	*Enzyme "activity"*	
			ATP-ase	*5′-nucleotidase*
0	2,200	1,300	1.4	3.4
2	1,150	525	1.5	0.2
4	314	490	1.0	—
6	$<$ 20	$<$ 50	0.4	—
2 (+ 0.015 M Mg^{++})			1.9	0.3
2 (+ 0.015 M Ca^{++})			0.8	0.2

* Measured on TCA extract.

Dialysis of venom against 0.08 M veronal buffer, pH 8.4 effected 79 % enhancement of the 5′-nucleotidase. Zinc sulphate (47 μM) neutralised this effect. Mn^{++}, Co^{++} and Mg^{++}, in decreasing order, raised the activity further. Ca^{++} inhibited slightly, confirming a previous report[3] (see Table III). Such dialysis hardly affected the ATP-ase (Table IV). Dialysis at the same pH against 0.008 M buffer increased the 5′-nucleotidase activity (in 0.08 M buffer) to 9.3 (at pH 8.4) and 16.0 (at pH 7.1)

TABLE III

EFFECT OF CERTAIN METAL IONS ON 5′-NUCLEOTIDASE

Conditions as for "buffer-dialysed" venom, Table IV.

No.	*Additions to reaction (Ultimate concn. mM)*	*"Activity"*
1	None	6.1
2	Mg^{++} (0.005)	6.1
3	Mg^{++} (0.047)	6.6
4	Zn^{++} (0.047)	3.6
5	Mn^{++} (0.005)	9.6
6	Co^{++} (0.005)	8.5
7	Ca^{++} (0.047)	5.8

Zn^{++} (47 μM) reduced these values to 6.6 and 12 respectively. Mn^{++} (5 μM) had no additional effect. Dialysis against tris buffer (0.01 M) at pH 8.5 resulted in a reduced

5′-nucleotidase activity (measured in 0.1 *M* tris) (10.4 at pH 7.4 and 3.9 at pH 9.0) compared with the undialysed venom. Mn^{++} (5 µ*M*) had little effect on this dialysed product. Zn^{++} (47 µ*M*) inhibited about 25 % at both pH values.

Both enzymes were unaffected by NaF (50 m*M*) but were completely inhibited by Cu^{++} probably indicating the involvement of amino groups (*cf.* [18] for alkaline phosphatase). ATP-ase was inhibited 50 % by sodium iodoacetate (45 m*M*).

Effect of chelating agents upon ATP-ase and 5′-nucleotidase: A preliminary experiment showed that dithizone increased the 5′-nucleotidase activity of the crude venom, while the ATP-ase was unaffected. 8-Hydroxy quinoline effected complete inhibition of both enzymes. The effect of various EDTA concentrations (8.4, 42, 420 and 4200 µ*M*) on the enzymes of crude and "buffer-dialysed" venom was determined (Table IV). After dialysis less EDTA was required for complete inhibition of both enzymes. This inhibition was reversed by $MgCl_2$ with considerable enhancement in the case of 5′-nucleotidase. Mg^{++} had a greater effect upon the ATP-ase after the "buffer-dialysed" venom: EDTA mixtures had been dialysed against equimolar veronal buffer (pH 8.4) overnight, even though it was found that such treatment would remove only about 50 % of the EDTA: metal complexes and less of EDTA itself (measured spectrophotometrically). (Table IV). The high Mg^{++} concentrations seemed essential for the re-activation of ATP-ase since dialysis overnight of "buffer-dialysed" venom: EDTA: magnesium ("280 m*M*") mixtures (Table IV, No. 15) against equimolar veronal buffer (pH 8.4) gave a low ATP-ase activity (0.35).

Mn^{++} had greater and Be^{++} less effect than Mg^{++} in overcoming EDTA-inactivation of 5′-nucleotidase. Ca^{++} had no effect with either enzyme (*cf.* [19]). Excess Mg^{++} and Mn^{++} inhibit 5′-nucleotidase. Zn^{++} inhibits the Mg^{++}-reactivated enzyme (Tables IV and V).

TABLE IV

EFFECT OF EDTA AND MAGNESIUM UPON ATP-ASE AND 5′-NUCLEOTIDASE

Incubation at 30° in 0.024 *M* veronal buffer, pH 8.4. Venom and "buffer-dialysed" venom, 0.11 %. AMP and ATP concentrations 5.6 m*M*.

No.	*Additions to reaction (Ultimate concn. mM)*	*5′-nucleotidase "activity"*		*ATP-ase "activity"*	
		Crude venom	*Dialysed venom*	*Crude venom*	*Dialysed venom*
1	None	3.4	6.1	1.4	1.3
8	EDTA (0.008)	—	7.3	—	1.9
9	EDTA (0.042)	3.7	6.5 (3.3*)	1.1	1.6
10	EDTA (0.42)	5.3	**0.3 (0*)	0.6	0
11	EDTA (4.2)	0	—	< 0.1	—
12	No. 10 + Mg^{++} (0.56)	—	2.2	—	0 (0.7***)
13	No. 10 + Mg^{++} (5.6)	—	14.0 (0*)	—	0.1 (0.9***)
14	No. 10 + Mg^{++} (28)	—	15.8	—	—
15	No. 10 + Mg^{++} (280)	—	4.1	—	1.4 (1.8***)
16	No. 11 + Mg^{++} (280)	12.2	—	1.0	—
17	No. 11 + Mg^{++} (2,800)	4.8	—	0.9	—

* After storage of EDTA-treated venom at — 20° for 21 days.
** Non-specific phosphatase activity < 0.05.
*** After dialysis of No. 10 overnight against equimolar veronal buffer, pH 8.4 at 20°.

After keeping the EDTA: venom mixtures at —20° for 21 days, the re-activation of 5′-nucleotidase by Mg^{++} was reduced, *e.g.* see Nos. 9 and 13 of Table IV.

Paper electrophoresis showed that the venom proteins are predominantly basic and the 5′-nucleotidase and ATP-ase activities move towards the cathode at pH 8–8.5. The electrophoretic pattern was not altered by the presence of EDTA.

TABLE V

EFFECT OF CERTAIN METAL IONS ON 5′-NUCLEOTIDASE INACTIVATED WITH EDTA

Conditions as for "buffer-dialysed" venom, Table IV.

No.	*Additions to reaction (Ultimate concn. mM)*	*"Activity"*
10	EDTA (0.42)	0.3
12	No. 10 + Mg^{++} (0.56)	2.2
18	No. 10 + Mn^{++} (0.37)	16.4
19	No. 10 + Mn^{++} (28)	3.4
20	No. 10 + Co^{++} (28)	0
21	No. 10 + Be^{++} (4.2)	8.8
22	No. 12 + Zn^{++} (0.042)	1.2

The inhibitory effect of specific antiserum upon the ATP-ase and 5′-nucleotidase has been reported previously[1]. A correlation was sought between the immunological and the enzymic changes effected under certain conditions.

LAMB[20] reported that after mixing the venom and antiserum, precipitate formation continued for 18–24 h. No parallel decrease in enzyme activity was observed. The reaction, with consequent enzyme inhibition, probably occurs rapidly while "mechanical" coagulation may take longer with little further effect on enzyme activity. CINADER found a decrease of 1–5 % for lecithinase[21] under such conditions but not for ribonuclease[22].

The effect of increasing amounts of antiserum on enzyme activity is shown in Table VI. LAMB[20] found that precipitate formation was maximum at an antiserum/venom ratio of 500/1 (by wt.). The ATP-ase inhibition may parallel the precipitate formation but ratios above 100 were not tried.

TABLE VI

INHIBITION OF ATP-ASE AND 5′-NUCLEOTIDASE BY SPECIFIC ANTISERUM

Incubation at 30° in 0.041 *M* veronal buffer, pH 8.4; 0.14 % venom; 5.0 m*M* AMP or 2.6 m*M* ATP.

Antiserum/g venom (g)	*% Inhibition*	
	5′-nucleotidase	*ATP-ase*
0.25	35	—
0.5	74	—
1	86	20
2	93	—
4	95	49
8	97	—
16	98	—
100	99	79

From an antiserum–venom mixture (4:1 by wt.) left at 20° overnight at pH 8.4 the precipitate was removed by high-speed centrifugation. The supernatant contained 81 % of the ATP-ase and 15 % of the 5′-nucleotidase activity of the original mixture.

Assuming this to be uninhibited enzyme, the inhibition accompanying their incorporation in the precipitate is 96 % for 5′-nucleotidase and 70–75 % for ATP-ase (allowing for inhibition due to AMP accumulation[23]). However, CINADER[21] has reported a loss of activity upon centrifuging a mixture of lecithinase and antiserum, so the calculation may be invalid.

Both enzymes were inactivated by heating at 75° for 30 min. Shorter periods showed the 5′-nucleotidase to be the more heat-stable at pH 8.4. After 1 min it was 91.5 % inhibited—(ATP-ase, 99.6 %); after 5 min, 98.8 %; after 20 min, 99.5 %. Similar results were obtained at pH 7.0. Since LAMB[20] found that the non-heat coagulable proteins of the venom would form an undiminished amount of serological precipitate, it would seem that the two enzymes in question cannot make a large contribution to the precipitate volume.

Hydrolysis of inosinephosphates

The action of the venom on ITP (and IDP) was similar to that on ATP (and ADP). In veronal buffer at pH 8.4, the ITP-ase activity rose above that of the 5′-nucleotidase so that IMP accumulated during the breakdown of ITP. Specific immune serum (× 2 wt. of venom) inhibited these activities to about the same extent as those of the adenosine phosphatases, the 5′-nucleotidase "activity" being reduced to 0.09.

Hydrolysis of pyridine nucleotides

DPN breakdown at the *pyro*-P bridge by snake venoms has been reported[24,25] and has been confirmed for cobra venom by BHATTACHARYA[26] and the present work (Table VII). The NMN produced is hydrolysed at 1.8 % of the rate for AMP which is comparable with the results of HEPPEL AND HILMOE[3] for other snake venoms. Antiserum (× 4 wt. of venom) decreased the DPN-ase activity by 60 %. Part of this inhibition was due to AMP accumulation[23]. (*cf.* ATP-ase). Low EDTA concentrations had little effect upon the DPN-ase activity of dialysed venom but 420 μM EDTA caused 93 % inhibition. Mg up to 5.6 mM did not reverse this inhibition but 280 mM Mg effected 100 % enhancement. (*cf.* ATP-ase). The pH optimum of the crude-venom DPN-ase was in the region of pH 9.0 or above. (Fig. 1). For the purified enzyme from

TABLE VII

HYDROLYSIS OF DPN BY COBRA VENOM

Incubation at 30° in 0.016 *M* veronal buffer, pH 8.4. "Buffer-dialysed" venom, 0.11 %.

Substance	*Concentrations (mM) after various times*			
	0 min	*5 min*	*10 min*	*15 min*
DPN	4.51	3.39	2.71	2.11
(AMP + NMN)*	0.45	2.16	2.86	3.37
NMN**	0	1.10	1.85	2.38
AMP (calc.)	0.45	1.06	1.01	0.99
Ortho-P	0.41	1.01	1.64	2.29
Adenosine	1.40	1.93	2.75	2.98

* From phosphate analysis.
** From nicotinamide analysis.

Maine potatoes, KORNBERG AND PRICER[27] found a broad optimum at pH 6.5–8.5 with 50 % reduction at pH 9.0.

The ATP-ase and DPN-ase are inhibited by the other's substrate and there is no summation of rates of hydrolysis. However, the kinetics preclude any possibility of a single enzyme acting on both substrates. With ADP the inhibition is not mutual, ATP-ase only being affected. There is no summation of rates and the kinetics suggest a single enzyme. However, supporting evidence is required especially in view of the discovery of specific nucleoside-5′-diphosphatases[28, 29].

Hydrolysis of coenzyme A

Incubation for 18 h (veronal buffer, pH 8.4) with cobra venom effected an almost complete degradation of coenzyme A (as measured microbiologically[30] by Dr. W. S. PIERPOINT) and chromatography showed that the coenzyme had been replaced by two spots, R_F values 0.024 and 0.063. Pantotheine-4-phosphate corresponded with the lower one. *Ortho*-P and adenosine appearing were accounted for by the "AMP" impurity which disappeared after incubation. The upper spot, therefore, which contained twice as much phosphorus as the lower, was probably adenosine-3′,5′-diphosphate suggesting that cobra venom hydrolyses coenzyme A at the *pyro*-P bond without subsequent liberation of *ortho*-P (*cf.* [31]).

No hydrolysis of TPP was apparent after 18 h incubation at pH 8.4.

Hydrolysis at pH 8.4 of FMN and R-5-P was slow ("activities", 0.013 and 0.031 respectively) (*cf.* [3]). The overall effect of EDTA (0.42 m*M*) plus Mg^{++} (5.6 m*M*) on the "buffer-dialysed" venom was 44 % inhibition of FMN hydrolysis and 140 % enhancement of R-5-P breakdown tending to confirm the suggestion[3] that different enzymes are involved.

DISCUSSION

The pH optima found for 5′-nucleotidase fall roughly into two categories: (a) values around 7.0, *viz.* for dialysed extracts[2, 32] of nerve tissue (7–7.5), human sperm (6.3), and other human tissues (7.0); (b) values of 8.5–8.9 for the partially purified enzyme of certain snake venoms[9] and bull seminal plasma[3] and for whole bull seminal plasma[23]. The value found for the cobra venom enzyme falls easily into the former category. Sources in this category only contain significant amounts of non-specific monophosphatases. The snake venoms studied by GULLAND AND JACKSON[9] for 5′-nucleotidase were selected for their lack of non-specific enzyme, and in bull seminal plasma the non-specific phosphatase activity is not more than 0.4 % of that of 5′-nucleotidase. The significance, if any, is unknown.

Enhancement of the 5′-nucleotidase activity after dialysis of the venom against veronal buffer would seem to be due to removal of inhibitory zinc ions, since re-addition of these reduces the activity to its original value. Zinc inhibition of 5′-nucleotidase has been reported previously[33, 34], and since it is observed at both pH 7.0 and 8.4 it is likely to be a true inhibitory effect upon the enzyme and not due to a shift of the pH optimum, as reported for acid and alkaline phosphatases[35]. The greater degree of enhancement obtained using more dilute buffer for dialysis is difficult to explain especially in view of the inhibition shown on dialysis in tris buffer. It cannot be due to an increased zinc removal, since re-addition does not effect a corresponding inhibition. The greater inhibition resulting from dialysis against water at room temperature compared with that at 0° must be ascribed to a combined effect of temperature and ion-removal, since the undialysed enzyme is stable when stored at room temperature at similar pH values. That the enzyme activity is susceptible to ion concentration is shown by the different effects of dialysis against water and 0.9 % KCl at 0°. The partial precipitation of the enzyme and possible denaturation effects during water-dialysis may be partly responsible for this difference.

The influence of manganese on the 5′-nucleotidase of the dialysed venom is contrary to the finding for the bull-semen enzyme[3], where manganese could not replace magnesium, but is in accordance with the recent findings of REIS[34] for the aorta and placenta enzymes (compare also for swine kidney alkaline phosphatase[36]). The effect of cobaltous ion confirms ZELLAR's activation effect[37] and may accord also with REIS's inhibition effect at higher cobalt concentrations[34]. As neither manganese nor cobalt were found in the venom, magnesium remains as a possible naturally-occurring activator. This is supported by the ready reversal of EDTA-inactivation of the enzyme by magnesium ions. However, that this is effected less readily after a prolonged period remains to be explained. Two possibilities arise: (a) denaturation of the enzyme by the EDTA, which may occur without significant change in the mass:charge relationship, and (b) removal of a bound metal ion from the enzyme molecule. There is evidence that venom contains a moiety having bound magnesium since part is not liberated by trichloracetic acid extraction. Furthermore, magnesium was found to accompany buffer-dialysed venom through a buffered, Dowex-50 column under conditions which removed all ionic magnesium[23]. However, denaturation of 5′-nucleotidase by EDTA seems a possibility.

The need for a large excess of magnesium ion for reactivation of EDTA-inactivated ATP-ase and DPN-*pyro*phosphatase suggests that this process is more complex than can be accounted for by mere chelation of EDTA with magnesium. The increased effect of even the highest magnesium concentration after dialysis of the EDTA-venom mixture is difficult to explain unless one assumes a fairly strong combination between enzyme and EDTA. The enzyme may be considerably more sensitive to EDTA than is 5′-nucleotidase. A denaturation by EDTA may be reversible by these high magnesium concentrations. The nature of the magnesium, or other activating influence associated with these enzyme activities warrants further study.

The greater incorporation of the 5′-nucleotidase into the serological precipitate may not mean that it is a more powerful antigen than the ATP-ase but merely that there is more of it in the venom, effecting greater anti-enzyme production. Heat-inactivation is likely to have sufficiently drastic effects on enzyme configuration to make precipitate formation with the anti-enzyme unlikely and since LAMB[20] failed to detect a reduction of serological precipitate after heat-coagulable proteins were removed, it is unlikely that these two enzymes play a major role in its formation. BRAGANCA AND QUASTEL[38] ascribed the toxic principle of cobra venom to a heat-stable enzyme; a lecithinase. This activity would disrupt cell-wall and particulate structures and, alone, would probably be sufficient to be lethal. However, many of the vital cellular cofactors would thus become available to the venom enzymes and would be degraded, *e.g.* ATP, DPN, coenzyme A, uridine-diphosphate-glucuronic acid[39] and probably others. This latter effect may be subsidiary to that of the lecithinase and may not affect significantly the number of experimental animals that are killed, in which case, heat-inactivation would appear not to alter the toxicity of the venom. The toxicity of snake venoms is now thought to be made up of several "activities", *e.g.* haemolytic, neurotoxic. The separation of these two by electrophoresis has been reported[40]. It would be of interest to know of their respective heat-stability, and enzymic properties. It was reported that the latter "toxin" is a small, dialysable molecule.

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ADENYLATE KINASE OF MAMMALIAN ERYTHROCYTES

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SUMMARY

The various blood constituents have been shown to be devoid of adenylate kinase activity, with the exception of the erythrocytes, where the enzyme activity is not bound to the stromal fraction. The optimal H^+ concentration is at pH 7.5. Strong Mg^{++} activation and rapid denaturation by heat were found, while no appreciable inhibition by NaN_3 was observed. The enzyme does not sediment when centrifuged for 210 min at 170,000 × *g*.

The preparation contains an enzyme that specifically catalyzes the reversible transformation 2 ADP $\rightleftharpoons$ ATP + AMP and is devoid of other appreciable nucleotide monophosphate kinase, ATPase and ADPase activity.

INTRODUCTION

Studies on phosphorus metabolism have revealed the presence in red cells of the enzymes of the glycolytic pathway leading to adenosinetriphosphate synthesis[1] and several ATPases[2]. Similar enzymes have been described in leucocytes[3], and ATPase has been found in blood platelets[4].

Although adenylate kinase activity (2 ADP $\rightleftharpoons$ ATP + AMP) has been reported in human erythrocytes by OVERGAARD HANSEN[5], a thorough study of this enzyme, either in whole blood or in its separated constituents, has never been made.

A study of adenylate kinase activity of blood consituents and some properties of the enzyme are reported here.

EXPERIMENTAL

Materials and methods

Chromatographically pure ATP and ADP were prepared from the commercial products by chromatography on Dowex 1-X2, 200–400 mesh, according to COHN AND CARTER[6]. The eluted substances were freed from inorganic salts by adsorption on activated Norite and elution with H_2O–ethanol–NH_3 (54:45:1) as previously described[7]. Ammonia, alcohol, and part of the water were removed by concentrating *in vacuo*.

The following abbreviations are used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine 5′-monophosphate; ITP, inosine triphosphate; IDP, inosine diphosphate; IMP, inosine monophosphate; P_i, inorganic orthophosphate; Hb, hemoglobin; TCA, trichloroacetic acid.

ITP, IDP, IMP were the crystalline Na salts, kindly donated by Sigma. IDP was completely purified by ion exchange chromatography according to DEUTSCH AND NILSSON[8] and adsorption–elution on Norite[7].

Unless otherwise stated, the reaction mixture contained in a final volume of 0.5 ml, 2 m*M* ADP, 0.014 *M* diethylbarbiturate buffer of the desired pH, and an amount of the enzyme preparation measured by its Hb or protein content. The ratio of Hb (measured by the absorption of HbCO at 570 mμ) to protein measured by the biuret reaction[9] was usually 5:6.

The reaction was started by adding the enzyme preparation to the incubation medium, equilibrated at 37°. It was carried out for 8 min at 37° under constant agitation in a thermostated bath and was stopped either by heating for 2 min at 100° in boiling water or by addition of 0.1 vol. 50% TCA. The pH was checked before and after incubation. All experiments were carried out in triplicate. The samples were centrifuged and 0.1 ml of the clear supernatant was analyzed by descending paper chromatography in *n*-propanol–H_2O–100% TCA–NH_3 (75:20:5:0.5)[10], the separated compounds being determined by the orcine reaction after elution, as previously described[10].

Controls to which no substrate or enzyme had been added did not show formation of either ATP or AMP.

Enzyme preparations

Whole human blood was collected in four volumes of isotonic NaCl in order to prevent immediate clotting. Platelets were prepared according to DILLARD *et al.*[11]. Red and white cells were sedimented from diluted blood at 1,500 × *g* and washed three times with isotonic NaCl. By suspending in bidistilled water to about 12% Hb, hemolysis was produced and leucocytes were sedimented at 1,500 × *g* for 20 min. After further dilution to 5% Hb, the stromal fraction was sedimented at 18,000 × *g* for 45 min, separated from the supernatant "hemolysate", and washed twice with water. A cytological control confirmed the homogeneity of each preparation. Serum was obtained from undiluted blood after clotting.

RESULTS

The various blood constituents were assayed separately after adenylate kinase activity had been found in whole blood (10 mg Hb per samples). Reaction mixtures contained 12 mg protein for assays on white cells, platelets and stromas of erythrocytes and 4 mg for serum.

No activity was shown by either of these preparations; adenylate kinase activity was found, however, in hemolysed total erythrocytes (10 mg Hb per sample) and in the "hemolysate". Stromal free "hemolysate" was, therefore, used in all further experiments. Adenylate kinase activity, comparable with that of human cells, was also found in horse, pig and ox erythrocytes.

Purity of the preparation

In the following sections the results are reported as μmoles of ATP and AMP formed, and μmoles of residual ADP. It will be seen that the amounts of ATP and AMP do not always strictly coincide, as the stoichiometry of the reaction would require. Differences are not large, however, and the recovery (ATP + AMP + ADP) always averages 100%. ATPase and ADPase were assayed in 0.014 *M* diethylbarbiturate buffer on 2 m*M* ATP or ADP at pH 8.5 with 5 mg Hb and at pH 7.6 with 0.5 mg Hb and $9.5 \cdot 10^{-4}$ *M* Mg^{++}. No P_i was detected when directly assayed[12], and chromatography confirmed that there was no breakdown of ATP and only adenylate kinase activity was detectable on ADP. Nucleotide monophosphate kinase activity was assayed in 0.014 *M* diethylbarbiturate buffer pH 7.6 and $9.5 \cdot 10^{-4}$ M$MgCl_2$ on 1 m*M* ITP and IMP, ITP and AMP, ATP and IMP, and on 2 m*M* IDP, using 0.5 mg Hb on a final volume of 0.5 ml. No phosphate transfer or dephosphorylation were detected by chromatographic analysis[10].

Thus, the homogeneity of our preparation as regards enzymes acting on adenosine or inosine nucleotides seems rather satisfactory, since, as will be seen from subsequent sections, glycolytic and oxidative phosphorylations, ATP–P_i exchange and deamination of ATP and ADP can also be excluded.

Reversibility of the reaction

The reversible interconversion 2 ADP $\rightleftharpoons$ ATP + AMP was demonstrated by parallel experiments on samples containing either ADP or ATP + AMP as substrate. Conditions and results are shown in Table I.

TABLE I

REVERSIBILITY OF THE ADENYLATE KINASE REACTION IN HUMAN ERYTHROCYTES

Assay medium: 0.014 *M* diethylbarbiturate buffer pH 7.4, $9.5 \cdot 10^{-4}$ *M* $MgCl_2$, substrate see below, 0.1 ml hemolysate containing 0.5 mg Hb. Final volume 0.5 ml; 8 min at 38°; reaction stopped by addition of 0.1 vol. 50% TCA.

Substrate	*Formed (µmoles)*	*Residual (µmoles)*	*Total recovery (µmoles)*
1 µmole ADP	ATP 0.15 AMP 0.19	ADP 0.65	0.99
0.5 µmole ATP + 0.5 µmole AMP	ADP 0.40	ATP 0.30 AMP 0.31	1.01

pH optimum

The pH/activity curve is shown in Fig. 1. A clear optimum is found at pH 7.4–7.6. When greater amounts of enzyme preparations were used (above 10 mg Hb), formation of IMP was observed from pH 6 to pH 7.4. No other adenine or inosine nucleotides or nucleosides or free purines could be detected at any pH.

Thermal stability

The inactivation of the enzyme by heat was studied by adding a volume of the hemolysate to twice its volume of water, previously equilibrated at the desired temperature in a water bath. The heating was continued under constant agitation, and aliquots were taken at regular intervals. These were immediately cooled on a refrigerated plate and subsequently used for determining the residual activity. Results are shown in Table II. Preparations heated in 0.1 *N* HCl were compared with an unheated hemolysate 0.1 *N* with respect to HCl.

Heating at 100° in water (pH 6) or in 0.1 N HCl completely destroys the enzyme activity in 2 min, while on heating at 50° in water about 50 % of the original activity is retained even after 30 min preincubation.

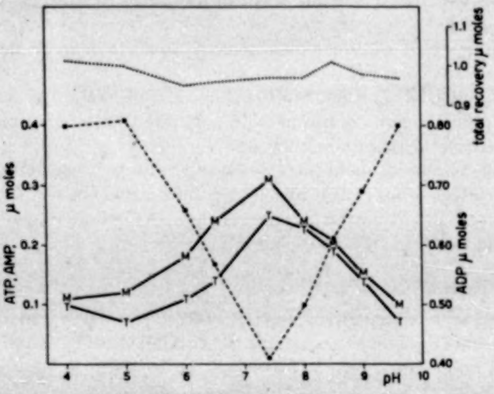

Fig. 1. pH/activity curve of erythrocyte adenylate kinase. 2 mM ADP, 0.014 M diethylbarbiturate buffer of the desired pH, 0.1 ml hemolysate containing 3 mg Hb. Final volume 0.5 ml; 8 min at 38°; reaction stopped by addition of 0.1 vol. 100 % TCA. ——— µmoles ATP (T) and AMP (M) formed; ------ µmoles ADP residual; ······ total recovery in µmoles.

TABLE II

THERMAL INACTIVATION OF HUMAN ERYTHROCYTE ADENYLATE KINASE

Assay medium: 0.014 M diethylbarbiturate buffer pH 7.6, 1 µmole ADP, 0.1 ml hemolysate containing 5 mg Hb. Final volume 0.5 ml; 8 min at 38°; reaction stopped by addition of 0.1 vol. 50 % TCA.

Preheating at 50° (minutes)	*Formed (µmoles)*		*Residual (µmoles)*	*Total recovery (µmoles)*
	ATP	*AMP*	*ADP*	
0	0.24	0.22	0.50	0.96
5	0.13	0.16	0.74	1.03
15	0.11	0.15	0.74	1.00
30	0.12	0.15	0.72	0.99

Activators and inhibitors

The effect of increasing Mg^{++} concentration was determined on samples containing an amount of enzyme preparation corresponding to 0.66 mg Hb. The activity increases gradually: at optimum concentration ($9.5 \cdot 10^{-4}$ M $MgCl_2$) it is six times as great as when no $MgCl_2$ is added. At higher concentrations (maximum $MgCl_2$ concentration assayed $3.6 \cdot 10^{-3}$ M) it is slightly less than the optimum.

The effect of 0.002 M NaN_3 was studied under the conditions described in a previous section for ATPase and ADPase activity. A decrease of ATP formation is observed; it averages 25 % at pH 8.5, 3 mg Hb, no $MgCl_2$ added, and 18 % at pH 7.6, $9.5 \cdot 10^{-4}$ M $MgCl_2$, 0.5 mg Hb.

Effect of different buffers

Activities in 0.03 *M*, 0.014 *M* mono and disodium phosphate, 0.014 *M* diethylbarbiturate and tris buffers pH 7.6 were compared at optimum Mg^{++} concentration and 0.5 mg Hb. With respect to diethylbarbiturate, the ATP/AMP ratio increases by 5 % in 0.014 *M* phosphate, decreases by 16 % in 0.03 *M* phosphate and by 34 % in trishydroxymethylaminomethane. In order to ascertain whether P_i was added to ADP in other ways than by adenylate kinase activity, the experiment with 0.014 *M* phosphate buffer was repeated with addition of 0.1 μC $^{32}P_i$.

No labelling was found in ATP, ADP or AMP, either by direct counting or by autoradiography. These results rule out the possibility, under our experimental conditions, of ATP formation in the glycolytic or oxidative pathway, or an ATP $\rightleftharpoons$ P_i exchange.

Ultracentrifugation

The hemolysate was centrifuged for 210 min at 39,500 rev./min in a Spinco Model L ultracentrifuge, swinging rotor SW 39. No boundaries were observed in the centrifuged fluid: the upper portion of supernatant (1 ml, average centrifugal force 90,000 × *g*) was collected separately from the rest (170,000 × *g* at bottom). A very small precipitate (P) was obtained and assayed after suspension in water without washings. Each sample contained 0.6 mg protein and $9.5 \cdot 10^{-4}$ *M* $MgCl_2$.

The specific activities of both fractions of the supernatant are the same as those of the uncentrifuged hemolysate, while in the precipitate only ADPase activity is found. Since stromas had been centrifuged off the hemolysate used for this experiment at 15,500 × *g* for 30 min, it is likely that fraction P is due to unsedimented stromal residues.

DISCUSSION

Like myokinase, the adenylate kinase of erythrocytes is specific for the reaction 2 ADP $\rightleftharpoons$ AMP + ADP.

Bowen and Kerwin[13] found that the optimum of resin-purified muscle enzyme is about pH 8 and that it is shifted to lower values by Mg^{++} concentrations higher than $3 \cdot 10^{-4}$ *M* ([Mg] to [ADP] ratio = 0.6). In our experiments, the medium used for determining the pH/activity curve was probably about $4 \cdot 10^{-5}$ *M* with respect to Mg^{++} owing to the endocellular Mg[14] dissolved in the hemolysate ([Mg] to [ADP] ratio = 0.02): it seems very unlikely that this Mg concentration would influence the pH optimum.

The activation by Mg^{++} is also much greater than that found for myokinase by Bowen and Kerwin[13]. They observed that the heat stability of the muscle enzyme decreases during purification; Mg^{++} 10^{-6} and 10^{-3} *M*, NaCl, albumin[15] and actomyosin[16] partially protect the enzyme against heat inactivation. Our data are consistent with those of Bargoni[15] on the protective effect of Mg^{++} on myokinase; they show that Hb does not have the protective effect on the enzyme of erythrocytes that it has on myokinase[17]. The results of ultracentrifugation suggest that the adenylate kinase of erythrocytes has a relatively low molecular weight (crystalline myokinase has a molecular weight of 21,000[18]). In the light of these findings, it is reasonable to suppose that in the "hemolysate" adenylate kinase is found in a higher degree of purity than is the enzyme of unpurified muscle extracts.

ACKNOWLEDGEMENT

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AN EFFECT OF THE SUBSTRATE ON UREA-MODIFIED PENICILLINASE

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SUMMARY

α-Penicillinase retains full activity when exposed simultaneously to the substrate and to 0.0025 M iodine. Urea-modified (γ-type) penicillinase is inactivated under such conditions. Preincubation with the substrate causes a shift from γ- to α-type, as measured by change in reactivity with iodine. The shift is not reflected in the rate of the enzymic reaction. The significance of the substrate-induced change is discussed.

INTRODUCTION

Exopenicillinase of *Bacillus cereus* can exist in two antigenically different states, which also differ in their reactivity towards iodine[1]. The transition from one state to the other can be brought about in a number of ways, including exposure to hydrogen-bond breaking agents, such as urea[2] or guanidine hydrochloride[3]. The resulting modification of penicillinase does not, however, seem to affect its enzymic activity. Thus the molecular activities of the original (α-type) and of the urea-modified (γ-type) enzyme are practically identical[2].

It appeared at first as if the properties examined reflected configurational changes not involving regions the integrity of which was essential for enzymic function[1]. Yet, though not impossible, it was difficult to visualize a situation where treatment known to break hydrogen bonds and shown to result in exposure of previously unreactive groups would leave the enzymic activity of the molecule completely unimpaired. We therefore suggested an alternative interpretation, whereby the enzyme molecule is restored to the original α-configuration through contact with the substrate[2].

The behaviour of urea-modified penicillinase in the presence of the substrate has now been examined and the data to be presented seem to favour the latter interpretation.

MATERIALS AND METHODS

α-penicillinase. A preparation of penicillinase was obtained from the culture supernatant of *Bacillus cereus*, mutant strain 569/H. This mutant strain, derived[4] from the inducible penicillinase forming *Bacillus cereus* NRRL 569, produces penicillinase constitutively. The culture was started from spores inoculated into a medium consisting of Difco Peptone (1 %) Bovril Meat Extract (0.3 %) and NaCl (0.2 %) and prepared according to POLLOCK[5].

After reaching logarithmic phase the cells were transferred to a casein–hydrolysate–citrate medium (CH/C) prepared according to KOGUT *et al.*[4] and shaken at 35° until enzyme production began to level off. The supernatant enzyme was concentrated by adsorption and purified by a procedure described elsewhere[3] or by the method of KOGUT *et al.*[4]. In either case the specific activity of the penicillinase preparation used was close to that found for the crystalline preparation of α-penicillinase[4] of *B. cereus* 569. Such preparation will be referred to as α-penicillinase. It was stored at −5°, and all dilutions were made in 0.5 % gelatin unless otherwise stated.

Urea-modified penicillinase. As shown previously[2], α-penicillinase becomes sensitive to 0.0025 *M* iodine if preincubated with 5 *M* urea. Preincubation is not necessary if gelatin is omitted from the system, and most of the enzyme is instantaneously inactivated by the iodine in an aqueous solution of urea[3]. Thus penicillinase assayed under conditions in which the gelatin solution has been replaced by a 5 *M* urea solution will be referred to as urea-modified penicillinase.

Penicillin and penicilloic acid. The penicillin used was the sodium salt of benzylpenicillin (Merck). Penicilloic acid was prepared by alkaline cleavage of penicillin[6]. 1 ml of a solution of benzylpenicillin, containing 200,000 units, was treated with 1 drop of 20 % NaOH for 20 min at room temperature. It was then diluted 1:40 in 0.1 *M* phosphate buffer pH 7. Such a preparation was found to bind an amount of iodine equivalent to that bound by an enzymically cleaved preparation.

Enzyme assay. The iodometric procedure for assay of α-penicillinase has been described[1,3]. In that procedure the α-type activity is determined by the time required for a complete decolorization of a known amount of iodine present in the assay mixture. The decolorization is due to the formation of a colourless complex between the iodine and the reaction product (penicilloic acid). The iodine concentration at the beginning of the reaction is sufficient to inactivate completely the immunologically distinct γ-penicillinase[7]. It also inactivates reversibly modified penicillinase, said to be in the γ-state[1,2].

Modifications of the standard procedure will be dealt with in the next section.

RESULTS

Assay of α-penicillinase

A. *Variation of time of iodine addition.* The following procedure has been adopted for assaying changes in the iodine-sensitivity of penicillinase in the course of the enzyme reaction. The reaction is started by introducing 150–200 units of penicillinase in 0.2 ml into an assay mixture which consists of 3 ml of the assay medium, *e.g.* 0.5 % gelatin solution, and 1 ml of 0.1 *M* phosphate buffer pH 7 containing 5000 units of penicillin. 0.5 ml of iodine solution of 0.025 *M* I_2 in 0.125 *M* KI aqueous solution is added after a specified time interval. The time required for a complete decolorization is noted, and the activity calculated as in the standard procedure[1]. It will be realized that since the penicilloic acid formed combines instantaneously with its equivalent of iodine, the end point of the decolorization in the case of α-penicillinase should be independent of the time of addition of iodine.

The first set of measurements consisted of assays of α-penicillinase in 0.5 % gelatin with the iodine added at 30 sec intervals. As can be seen from Table I (columns A, B) the decolorization time is in fact independent of the time of addition of iodine. Columns C, D (Table I) demonstrate that the accuracy of the iodometric assay is not impaired by the procedure of adding iodine in the course of the reaction.

TABLE I

ASSAY OF α-PENICILLINASE WITH VARYING TIMES OF ADDITION OF IODINE

A *Time before iodine addition (min)*	*B* *Decolorization time (sec)*	*C* *Activity (units/ml)*	*D* *Deviation from mean value %*
0.0	311	187.5	+5.6
0.5	369	160.0	−9.8
1.0	360	161.3	−9.1
1.5	342	172.5	−2.8
2.0	312	187.0	+5.4
2.5	310	187.5	+5.6
3.0	300	194.0	+9.4
3.5	311	187.5	+5.6
4.0	314	186.3	+5.0
4.5	344	170.5	−3.9
5.0	352	166.8	−6.0

B. *Variation of iodine concentration.* A progressive delay in addition of iodine to the assay system should be in principle equivalent to the exposure of the enzyme to progressively decreasing concentrations of iodine. This is because before the addition of the iodine the enzyme produces an amount of penicilloic acid proportional to the time of delay; penicilloic acid combines instantaneously with the added iodine and reduces its effective concentration to a value inversely proportional to the time of delay.

In order to test the validity of the iodometric procedure for various iodine concentrations a second series of readings was taken with iodine present at zero time. The variable in this set of assays was the initial concentration of iodine. Should α-activity be unaffected by the iodine concentrations tested, the decolorization time would be directly proportional to the amount of iodine in the assay system. The results are summarized in Table II. By multiplying the time of decolorization by the factor of iodine dilution we obtain the time required for complete decolorization of the amount of iodine used in the standard assay. The corresponding activity is then read from the standard calibration curve.

It is obvious from Table II that the activity of α-penicillinase is completely unaffected by an initial exposure to any concentration of iodine within the range tested.

TABLE II

ASSAY OF α-PENICILLINASE WITH VARYING CONCENTRATIONS OF IODINE

A *Iodine concentration* *(× 10^{-4} M)*	B *Decolorization time* *(sec)*	C $\left(\frac{B}{A} 25 \cdot 10^{-4}\right)$* *(sec)*	D *Activity* *(units/ml)*	E *Deviation from mean value* %
25.0	309	309	190.0	−3.3
22.5	267	297	197.5	+0.5
20.0	240	300	195.0	−0.8
17.5	220	315	186.5	−5.2
15.0	175	292	200.1	+1.8
12.5	140	280	208.8	+6.3
10.0	117	292	200.1	+1.8
7.5	92	306	192.5	−2.0
5.0	59	295	198.8	+1.2

* Time required for decolorization of the amount of iodine used under standard assay conditions (values of column B divided by those of A and multiplied by the standard iodine concentration).

The iodine sensitivity of penicillinase in urea

It follows from the previous paragraph that α-type activity is not affected by variations in the time of addition of iodine (set A) or in the initial concentration of iodine (set B).

In contrast, the activity of penicillinase in the γ-state is largely determined by these variables (see also ref.[8]). The question to be answered is whether the survival of γ-type activity as a function of exposure to iodine (sets A and B) is affected by previous contact with the substrate (set A).

The system studied here is that of penicillinase in urea. As shown previously[2], urea causes a shift from α-type to γ-type activity. In the following experiments the enzyme was assayed for sensitivity to iodine by both procedures described above except that the gelatin solution used in the standard assay was replaced[3] by a urea solution of 5 *M*. The concentration of the enzyme was adjusted so that the amount of penicilloic acid formed in 30 sec would be approximately sufficient to combine with $2.5 \cdot 10^{-4}$ *M* iodine. This relationship is expressed in the paired abscissae of Fig. 1.

Again one set of readings was taken with varying concentrations of iodine in the assay system. After correcting for the differences in decolorization time due to the variable amounts of iodine, a curve has been obtained which simply describes the survival of penicillinase in urea as a function of the iodine concentration to which it was initially exposed (Fig. 1, curve B). This curve will be compared with the one obtained from the other set of readings, in which the full amount of iodine is added at various times after the initiation of the enzymic reaction (curve A in Fig. 1).

It will be noted that curve A too represents the activity of the enzyme after exposure to iodine. (The assay was started—as before—upon addition of iodine, and the reduction in decolorization time due to preformed penicilloic acid has been corrected for).

As there was no difference in the conditions of the assay, both enzyme survival curves should overlap, unless previous contact with the substrate affected the subsequent reactivity of the enzyme with iodine.

In our case the "iodine delay" curve (Fig. 1A) is significantly different from the "iodine concentration" curve (Fig. 1B). While curve B shows a nearly linear dependence of the activity on the concentration of iodine, curve A shows a sharp increase in the iodine resistance of the enzyme as the reaction proceeds. Thus the enzyme was invariably found to become fully resistant to iodine before the reaction was half over, *i.e.* at the stage where the effective iodine concentration is sufficient to inactivate approximately half of the enzyme in urea.

Kinetics of the activity of penicillinase in urea

As shown in Fig. 1, the enzyme in urea becomes increasingly more iodine-resistant as the reaction proceeds. The problem arose whether the enzymic activity of the preparation was in any way affected by this change in reactivity with iodine. As pointed out in the introduction, this could be answered in the negative by considering the fact that the sum of α-type and γ-type activities was practically constant over a wide range of urea concentrations, and thus largely independent of the degree of the α- to γ-conversion.

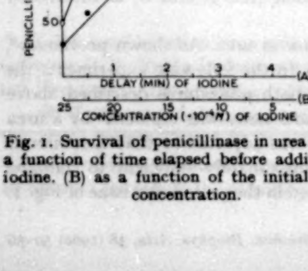

Fig. 1. Survival of penicillinase in urea: (A) as a function of time elapsed before addition of iodine. (B) as a function of the initial iodine concentration.

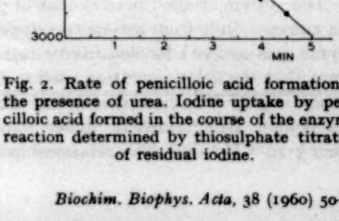

Fig. 2. Rate of penicilloic acid formation in the presence of urea. Iodine uptake by penicilloic acid formed in the course of the enzymic reaction determined by thiosulphate titration of residual iodine.

For a direct answer the actual rate of penicilloic acid formation in the presence of urea was determined. The assay of penicilloic acid was performed by a modification of a procedure suggested by PERRET[9].

A preparation of penicillinase in urea was allowed to react with the substrate under conditions identical with those of the "delayed iodine" assay. The reaction was stopped by the addition of 0.5 ml of a reagent containing 12.8 M acetic acid and 0.05 M iodine followed by 10 min incubation at 30°. By that time the enzymic activity was completely stopped and the penicilloic acid formed before the addition of the acetate reagent has combined with part of the iodine. The residual iodine was titrated against 0.0166 N Na-thiosulphate and the amount of penicilloic acid calculated from the difference. The results show that the formation of penicilloic acid is linear throughout, irrespective of the state of iodine sensitivity of the enzyme (Fig. 2).

Possible factors involved in the γ- to α-shift in the course of the enzymic reaction

The observed shift in the iodine sensitivity of the enzyme in urea could be ascribed to one or more of the following factors:

(a) contact with penicillin; (b) exposure to the accumulated penicilloic acid; (c) exposure to the phosphate buffer in which penicillin is delivered.

Factors (b) and (c) have been eliminated as a likely cause for the shift on the following grounds:

The effect of penicilloic acid. Penicilloic acid was prepared by alkaline cleavage of Na-benzyl-penicillin (see MATERIALS AND METHODS).

The enzyme preparation was preincubated for 5 min in 5 M urea. An amount of penicilloic acid just sufficient to combine with $5 \cdot 10^{-4}$ M iodine was added to the enzyme, (a) 60 sec before the beginning of the assay, (b) 60 sec after the beginning of the assay.

The iodine was added in both cases 60 sec after the beginning of the assay. At that stage the γ-type activity still forms a high proportion of the total (*cf.* Fig. 1) so that a two-minute contact with the penicilloic acid should cause a very marked increase in the activity surviving the exposure to iodine, if penicilloic acid were responsible for the shift.

As the readings obtained under conditions (a) and (b) were practically identical, it was concluded that the contact with penicilloic acid does not affect the iodine reactivity of the enzyme.

The effect of phosphate buffer. In the above assay procedure penicillin is delivered in 1 ml of 0.1 M sodium–potassium phosphate buffer, the function of which is to prevent acidification of the assay medium. The following experiment served to separate the possible effect of the phosphate from that of the substrate.

Penicillin was dissolved in distilled water at a concentration of 50,000 U/ml. 0.1 ml of that solution was used in the "5 M urea" assay. The phosphate buffer was added (a) 60 sec before the beginning of the assay, (b) 60 sec after the beginning of the assay.

The iodine was added in both cases 60 sec after the beginning of the assay.

As explained in the previous paragraph, the conditions were such that any effect of contact with phosphate on the iodine-sensitivity of the enzyme would cause a difference in values obtained under assay conditions (a) and (b), and as before, no such difference appeared.

DISCUSSION

In defining the changes observed in penicillinase-treated in a number of ways[1] we have adopted two independent criteria. One was the antigenic identity of the enzyme, as judged by its neutralizability by anti-α-penicillinase serum. The other was its reactivity with iodine, as judged by loss of activity through exposure to 0.0025 *M* iodine. Serological observations are difficult to interpret in experiments involving high concentrations of urea, so that the experimental evidence to be discussed rests mainly on measurements of changes in the iodine-sensitivity of the enzyme. While the iodine-sensitivity has not been shown to be sufficient to define the state of penicillinase with respect to antigenicity, it will be realized that wherever definable (*e.g.* in case of penicillinase pretreated with alkali or variously adsorbed[1]) the antigenic structure has been found to parallel the state of iodine-sensitivity. We also know that urea-modified penicillinase spontaneously reverts to the α-state, as judged by both criteria, when urea is removed[3].

We have shown that the actual iodine-sensitivity of penicillinase in urea (indicated by curve A in Fig. 1) deviates from the expected (Fig. 1, curve B) as the enzymic reaction proceeds. This deviation was found not to be due to contact with the penicilloic acid formed or with the phosphate buffer present. Thus by implication, it was through previous contact with the substrate that the enzyme acquired resistance to iodine.

Since we do not know of any other mechanism that would account for the observed changes, we suggest that the increase in iodine resistance in the course of the enzymic reaction represents a γ- to α-shift.

It will be remembered in this connection that the cell of *B. cereus* offers surfaces which by adsorbing the enzyme confer on it the properties of reversible γ-penicillinase[1]. A possible significance of a substrate-catalysed $\gamma \rightarrow \alpha$ shift in the context of the mechanism of penicillinase induction has to be borne in mind.

The experiments reported above were mainly designed to account for the observation that penicillinase seems to undergo configurational changes without any apparent impairment of its enzymic function. In our previous communication[2] we suggested a situation not unlike that observed in the case of ribonuclease[10], where the modified molecule reverts to the enzymically active conformation through contact with the substrate. While not conclusive, the present results are consistent with such an interpretation.

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IONIC EQUILIBRIA IN A PROTEIN CONJUGATE OF A SULFONAMIDE TYPE

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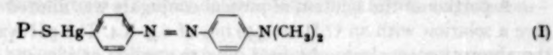
SUMMARY

The acidity constants of the $(CH_3)_2N$-group of 5-dimethylamino-1-naphthalene sulfonyl conjugates of glycine and of bovine serum albumin have been compared. In the native protein environment, pK_a is shifted by over 2.3 pH units. Denaturation of the protein by 8 *M* urea reduced the shift in pK_a to 0.9 unit. These and related observations are interpreted in terms of changes in the nature of the hydration lattice of the protein molecule.

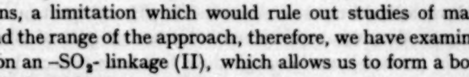
INTRODUCTION

It has been shown recently[1, 2] that some interesting features of the behavior of protein molecules may be followed in studies of the ionization equilibria of artificially conjugated complexes. The conjugate described previously was prepared from an azomercurial which combined with the protein through mercaptan side chains (I):



(I)

The use of a mercaptide linkage, however, confines one to combinations with sulfhydryl-containing proteins, a limitation which would rule out studies of many interesting systems. To extend the range of the approach, therefore, we have examined some conjugates depending on an $-SO_2-$ linkage (II), which allows us to form a bond with a lysine (or perhaps serine) residue:



(II)

The specific compound used, 5-dimethylamino-1-naphthalene sulfonyl chloride, was suggested by the work of WEBER[3] and of others[4, 5], who showed that this substance combines readily with several proteins under very mild conditions. The ionic equilibria of the $-N(CH_3)_2$-group in the conjugate so produced, (II), can be followed readily through changes in the absorption or the fluorescence of radiation. With conjugate (II), as with (I), acid-base equilibria have been found to change strikingly in the environment of a protein molecule.

EXPERIMENTAL

Preparation of conjugates

A solution of bovine serum albumin was prepared containing 80 mg of protein in 20 ml of 0.1 *M* Na_2HPO_4. To this cooled solution was added 1 ml of a cold acetone solution containing 2 mg of 5-dimethylaminonaphthalene-1-sulfonyl chloride. The mixture was kept in a refrigerator for 24 h. It was then placed in cellophane tubing and dialyzed in the cold first, for 18 h with shaking, against 0.1 *M* NaCl, secondly for 31 h against three successive portions of 0.01 *M* acetic acid and finally for several days against distilled water. At each stage the fluorescence intensity of the solution outside the dialysis bag was measured. It declined to a negligible value in the final dialysis, the amount coming off in the last stage representing less than 0.3 % of the dye originally added.

The moles of dye bound to the protein were computed from absorbance measurements at 340 mμ, plus the assumption that the extinction coefficient of $3.36 \cdot 10^6$ cm^2/mole found by HARTLEY AND MASSEY[6] for the chymotrypsin-dye conjugate is also applicable to albumin-dye conjugates. Preparations containing 4–11 moles of dye/mole of protein were obtained.

The glycine-dye conjugate was prepared by Mr. E. C. STELLWAGEN following the procedure of HARTLEY AND MASSEY[6] with minor alterations. The coupling was carried out in 0.5 *M* Na_2HPO_4 instead of 0.5 *M* NaOH. The twice recrystallized product had a m.p. of 158–9°, a yellow fluorescence, an absorption peak at 325 mμ at pH 9.1, and an R_F value of 0.81 in the butanol–formic acid–water solvent system of HARTLEY AND MASSEY[6]. All of these properties, except the absorption peak, agree with those reported[6]. The small discrepancy in position of the absorption peak is probably due to a typographical error.

Titration procedure

A portion of the solution of protein conjugate was diluted with distilled water to give a solution with an O.D. at 340 mμ of 0.3–0.4. The pH was then measured, and the absorption spectrum obtained over the region of 280–400 mμ. A portion of this solution was weighed to within 0.01 g, and then a measured quantity of hydrochloric acid was added from a microburet. The pH and spectrum of the solution were obtained. The procedure was repeated with further samples of solution and different quantities of acid (or base) until enough data had been collected to provide a smooth spectrophotometric titration curve. Suitable corrections were made for dilution of the solution by added acid or base.

The fraction, α, of the conjugate dye in the acid form was calculated by means of the equation[1]

$$\alpha = \frac{\text{O.D. soln. } \alpha - \text{O.D. basic soln.}}{\text{O.D. very acid soln.} - \text{O.D. basic soln.}} \qquad (1)$$

The pH's were measured with a Beckman meter model G.

Materials

Bovine serum albumin was a crystallized sample purchased from Armour and Co. The 5-dimethylaminonaphthalene-1-sulfonyl chloride was a purified grade obtained from the California Foundation for Biochemical Research. Glycine was C.P. grade of

H.M. Chemical Co. All acids, bases and salts were reagent grade. Acetone was Eastman Kodak spectroscopic grade.

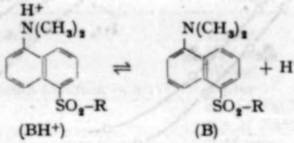
Optical measurements

The absorption of light was measured with the Beckman spectrophotometer, model DU, at about 25°. Fluorescence intensities were obtained with a fluorometric attachment to the Beckman spectrophotometer as described by FLETCHER, WHITE AND SHEFTEL[6a]. A solution of 2 mg of quinine sulfate in 100 ml of water was used as the reference.

RESULTS AND DISCUSSION

Computation of acidity constants

The equilibrium being studied may be represented by the equation



(2)

If R represents a small molecule, such as a glycine residue, $-NHCH_2COO^-$, then this dissociation can be described quantitatively by the familiar simple mass-law expression in logarithmic form,

$$\mathrm{pH} = \mathrm{p}K + \log \frac{(\mathrm{B})}{(\mathrm{BH}^+)} \tag{3}$$

A more convenient graphical representation of this equilibrium is a plot of α, the fraction of conjugate in the acid form BH^+, *vs.* the pH. A graph for the glycine conjugate is illustrated in Fig. 1. At the midpoint, $\alpha = 0.5$, $(BH^+) = (B)$, and hence the p*K* of the dimethylamino group is readily determined as 3.99 for the naphthalenesulfonylglycine conjugate.

When the dye is attached to a protein, eqn. (3) is no longer adequate to represent the ionization. Since the protein molecule carries a charge which varies with pH, the uptake of protons by the $(CH_3)_2N$-group will be affected electrostatically[7–9]. This electrostatic influence is generally treated by insertion of an additional term in eqn. (3) to give

$$\mathrm{pH} = \mathrm{p}K + \log \frac{(\mathrm{B})}{(\mathrm{BH}^+)} - 0.868\,\frac{\Delta F_{\mathrm{el}}}{RT}\,\bar{Z}_P \tag{4}$$

where ΔF_{el} is the electrical free energy change (per mole) for charging an assumed-spherical protein molecule uniformly with a total of unit charge and $\bar{Z}_P$ represents the mean net charge of the protein molecule.

Values of $\Delta F_{\mathrm{el}}/RT$, usually represented by w, may be computed from electrostatic theory[7–9]. However, since it has been clearly shown that such values cannot be used (without additional *ad hoc* assumptions) to describe the titration behavior of bovine serum albumin in acid solutions, we shall merely consider w as an experimental parameter and adopt numerical values for it which have been found by TANFORD, SWANSON AND SHORE[7] to fit the electrometric proton titration data.

To apply eqn. (4) to our spectrophotometric titrations of the albumin-dye conjugate, we must also choose values for $\bar{Z}_P$. To a first approximation we might assume that $\bar{Z}_P$ in solutions acid to the isoionic point is equal to r_H, the number of protons bound by serum albumin as we proceed from the isoionic point to the given pH. Values of r_H can be computed from the data of TANFORD, SWANSON AND SHORE[7]. Thereafter, if we adopt the assumption that the *intrinsic* affinity of the $(CH_3)_2N$-group

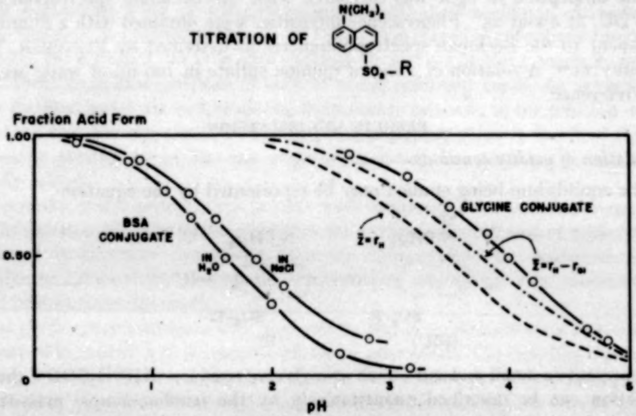


Fig. 1. Optical titrations of 5-dimethylaminonaphthalenesulfonyl conjugates of bovine serum albumin and glycine, respectively. Circles represent experimental points; — — — represents curve computed theoretically for the $-N(CH_3)_2$ group on serum albumin whose net charge $\bar{Z}$ equals r_H, the number of protons bound from the isoionic point to the given pH; — — — — represents theoretical curve correcting $\bar{Z}$ for the moles of anion, r_{Cl}, bound by the protein at the given pH. The theoretical curves are for protein in solutions of 0.1 ionic strength.

is the same whether the dye is attached to protein or to a small molecule such as glycine, we can compute readily $(B)/(BH^+)$ and hence α. Fig. 1 shows a titration curve calculated in this way for solutions with an ionic strength $\mu = 0.1$.

An alternative computation might also be made which takes cognizance of the fact that serum albumin binds anions, such as chloride, which are present during the titration. Thus we should write $\bar{Z}_P = r_H - r_{Cl}$, where r_{Cl} is the number of moles of chloride bound/mole of protein at the pH being considered. Some rough estimates of r_{Cl} may be made from the experiments of SCATCHARD, COLEMAN AND SHEN[8]. Again a titration curve for the albumin-dye conjugate may be calculated; this result also is illustrated in Fig. 1, again for solutions with $\mu = 0.1$.

Experimentally observed titration curves for a dye-albumin conjugate (containing a 5.9:1 dye to protein ratio) are also shown in Fig. 1. A small difference* is observed between the curve in the presence of 0.1 *M* NaCl and that in the absence of salt. Nevertheless both curves are displaced by a striking amount from the curves computed from electrostatic considerations.

For purposes of comparison we may define[1] as pK_a for the protein conjugate that pH at which (B) = (BH+), that is the midway point in the graph of *a vs.* pH (Fig. 1). Values of pK_a computed in this way are listed in Table I.

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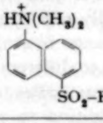
* The shift in pK_a of the protein conjugate in salt as compared to water is in the correct direction and of about the proper magnitude if the experimental values of TANFORD, SWANSON AND SHORE[7] are used for w and if the intrinsic pK_a of the $-N(CH_3)_2$-group is assumed to be the same in both solutions. However, this intrinsic pK_a is far lower than that for the glycine conjugate of the dye.

A few experiments were also run with conjugates containing different ratios of bound dye to protein. These are summarized in Table II. There is a slight upward trend in pK_a's in water with increasing mole ratio of dye: protein, but the differences are too small to be considered significant.

TABLE I

ACIDITY CONSTANTS OF  AT 25°

R	*Solution*	*pK*$_a$
–NHCH$_2$COOH	Water	3.99
	8 *M* urea	4.18
–OH	Water	4.27
–Bovine serum albumin (5.9/1)*	Water	1.67
	0.1 *M* NaCl	1.86
	0.1 *M* NaSCN	2.05
	8 *M* urea	3.27

* The numbers within the parentheses refer to the (average) number of dye molecules attached to each protein molecule.

TABLE II

VARIATION OF ACIDITY CONSTANTS WITH MOLE RATIO OF DYE: SERUM ALBUMIN IN CONJUGATE

Dye: protein	*pK*$_a$
4.1	1.60
5.7	1.64
5.9	1.67
10.9	1.70

*Nature of influence of protein environment on p*K$_a$

The fact that the pK_a of the $(CH_3)_2NH^+$- group is 1.67 when it is attached to the protein and 3.99 when it is on a small molecule in water shows that the protein environment has a very profound effect on the ionizing abilities of this group. The analysis outlined above indicates strongly that electrostatic effects are not responsible for this behavior. For several reasons, it seems unlikely, furthermore, that hydrogen-bonding between $(CH_3)_2\ddot{N}$- and a hydrogen donor group causes the shift in pK_a. The $(CH_3)_2\ddot{N}$- substituent of an aromatic ring contributes its pair of electrons to resonance with the ring and hence is not a good receptor of an H-bond. From the hydrogen-bonding viewpoint one would expect a much greater shift in the p*K* of $-COO^-$ groups of a protein since these are much stronger H-bond acceptors. In addition, in the conjugate of type (I), spectroscopic behavior is inconsistent with that observed[1] for related systems where hydrogen-bonding is present. Also as has been pointed out previously, it is difficult to see from a hydrogen-bonding viewpoint, why the addition of a long-chain anion should lower the pK_a of (I) even further.

As has been suggested earlier, the displacement of the titration curve of conjugate II (Fig. 1) toward lower pH's, *i.e.* toward greater H^+-ion concentrations, may be thought of advantageously as a "masking" effect. Thus a denaturing agent, such as urea, which can reveal naturally-occurring masked groups, also removes most of the unusual behavior of the artificially-produced masked groups. As is evident from Table I, the addition of urea to conjugate II raises pK_a to 3.27, a very substantial return toward the value of the dissociation constant of the $(CH_3)_2NH^+$-group attached to a small molecule. The effect of urea on conjugate II thus is similar to that on I.

Since a H^+–ion can rapidly reach the $(CH_3)_2N$- group on the protein, we have assumed that an aqueous passage is available to the proton, but since the basicity of the dimethylamino group is so different when it is attached to the protein, we have reason to assign some special properties to the water in the immediate neighborhood of the macromolecule. In addition to much evidence cited previously[1,2] for the assumption of a special ice-like character of regions of the hydrate water of proteins, we might also point to the isolation and structure determination of many crystalline hydrates of non-polar molecules[10,11], such as those of CH_4, C_3H_8, $(CH_3)_2O$, $(CH_3)_3CH$, C_6H_6, C_3H_7Br, CH_3SH, etc. It is apparent that a wide variety of non-polar molecules are capable of stabilizing water in crystalline structures containing large cavities (5–7 Å in diameter). In the absence of the non-polar molecules these structures would be unstable with respect to normal ice.

Such a direct demonstration of "icebergs" around non-polar molecules is not possible for dissolved solutes, but further indirect evidence is available. Among recent papers one might cite two dealing with thermal measurements. It has been shown by FRANK AND WEN[12], for example, that the apparent molal heat capacity, φC_{p_2}, of tetrabutylammonium ion in water solution is about 150 cal $mole^{-1}$ deg^{-1} higher than one might expect from additivity rules. Similarly GODDARD, HOEVE AND BENSON[13] have found that the heat capacity of octanoate ion is some 70 cal $mole^{-1}$ deg^{-1} higher in aqueous solution than one would expect from the behavior of the hydrocarbon chain in the liquid state. In both cases, the excess heat capacity has been attributed to the effect of the organizing of a solvent structure around the non-polar solute molecule. With a rise in temperature, some heat must be absorbed in the progressive thermal disorganization of the hydrate ice; it is this heat which contributes to the unexpectedly large $\bar{C}_{p_2}$ of these non-polar solutes.

In a protein molecule many non-polar side-chains exist in juxtaposition. Thus there could be a "coupling" of the ice lattices formed around each residue with a consequent strengthening of the resultant hydrate structure. It is of interest in this connection to recall the crystalline double-gas hydrates described by v. STACKELBERG AND MÜLLER[10] in which two non-polar molecules act cooperatively in stabilizing the aqueous crystal to a degree substantially greater than that possible with one type of solute molecule. The structure of the lattice formed by coupling of hydration water of protein side-chains may not be the same as that of the gas hydrates but the possibilities for cooperative interaction in the macromolecule should be even greater since the residues are even initially relatively fixed in position by the framework of the protein molecule.

The presence of patches or ribbons of ice-like coverings around various regions of a protein molecule would have both kinetic and equilibrium consequences.

Rates of diffusion of essentially all substances to a reactive site under an ice-layer would be greatly reduced. For ions the relative rates of diffusion in the presence and absence of the hydrate might be estimated from the values found by EIGEN AND DE MAEYER[14] for Li^+ in ordinary ice and water, $< 10^{-8}$ *vs.* $4 \cdot 10^{-4}$ cm^2 $volt^{-1}$ sec^{-1}. On this basis one can readily understand why Ag^+ titrates very slowly with –SH-residues in many proteins. Judging from the observation that mercaptans form crystalline hydrates[10], we might reasonably conclude that –SH-groups in proteins could participate cooperatively with neighboring side-chains in the formation of an ice patch. Diffusion of Ag^+ through this ice patch would be greatly hindered as compared to the movement of this ion in ordinary water. Consequently the protein mercaptan acts as if it were masked.

Hydrogen and hydroxyl ions, in contrast to all other substances, encounter no difficulties in traversing ice[14] because of the unique mechanism by which they can be transported. In the present studies of reaction (2), however, it is the equilibrium and not the rate[*] of ionization which is affected when the $(CH_3)_2N$-group is attached to protein. The shift in pK_a toward lower values means in essence that the environment is more favorable to the $(CH_3)_2N$ rather than to the $(CH_3)_2NH^+$-group. Such a bias seems very reasonable, for the creation of a charged group in place of the uncharged one would require some breakdown of the ice lattice of the protein. It is this requisite disorientation of the hydration lattice which obstructs the formation of the acid form of the amine.

Thus the behavior of the sulfonamide conjugate (II) parallels that of the mercaptide conjugate (I) studied previously[1,2]. Furthermore, in both systems, the ionization equilibria can be understood in terms of interactions of the protein, serum albumin, with solvent molecules. With this broadening of the range of available conjugates it should now be possible to compare the behavior of a series of proteins of different sizes and shapes.

ACKNOWLEDGEMENT

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[*] No special effort was made to examine the rate of proton uptake by fast-reaction techniques Within the few minutes required for mixing the solutions and taking an O.D. reading, equilibrium was attained.

THE TRYPSIN-CATALYZED HYDROLYSIS OF BENZOYL-L-ARGININE ETHYL ESTER

I. THE KINETICS IN DIOXANE-WATER MIXTURES*

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SUMMARY

The rate of hydrolysis of benzoyl-L-arginine ethyl ester catalyzed by trypsin has been measured in dioxane–water mixtures containing up to 88 volume percent dioxane. The initial rate of the reaction follows the requirements of MICHAELIS-MENTEN kinetics under all experimental conditions employed. The pH-variation of the rate at enzyme saturation in the mixed solvents is similar to that in water. At pH 8 the rate first increases as dioxane is added and then decreases at higher dioxane concentrations. The apparent MICHAELIS-MENTEN constant is a minimum at approximately pH 8, and increases with increasing dioxane concentration. The value at pH 8.6 in 88 % dioxane is 5500 times the value in water at this pH. Interpretations of various of the observed effects are suggested.

INTRODUCTION

In view of the fact that variations of solvent have led to important results in many kinetic studies of small molecules of known structure, it is of interest to apply this attack to an enzyme system. A hydrolytic enzyme was desired for a first study because solvent variation would alter the concentration of the essential reactant water. The trypsin-catalyzed hydrolysis of benzoyl-L-arginine ethyl ester (BAEE) has been shown[1] to be very convenient for kinetic measurements, and preliminary experiments indicated that it would be possible to obtain kinetic data with this system in dioxane–water mixtures up to high dioxane concentrations.

The effect of temperature and pH on the maximum rate of hydrolysis of BAEE in aqueous solution was investigated by GUTFREUND[2]. In the present paper we report the effect of pH, temperature and substrate concentration on the rate of the hydrolysis in dioxane–water mixtures.

A few experiments with hydrolytic enzymes in organic solvent–water mixtures have been previously reported. SCHWERT AND EISENBERG[3] found that the rate of the trypsin-catalayzed hydrolysis of benzoyl-L-arginine methyl ester is increased by the addition of ethanol up to a concentration of 32 volume percent, and that further

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[*] Contribution No. 1545 from the Sterling Chemistry Laboratory of Yale University.

[**] Adapted from Ph.D. thesis of T. INAGAMI, June 1958.

additions of ethanol cause a decrease in rate. The maximum rate observed was 50 % higher than the rate in water. Similar effects were observed to result from the addition of various other alcohols. On the other hand, KAUFMAN AND NEURATH[4] found that the rate, at enzyme saturation, of the chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosine amide was unaffected by methanol up to a concentration of 21 volume percent, although the apparent MICHAELIS-MENTEN constant for the system was nearly tripled. BARNARD AND LAIDLER[5] reported that the saturation rate of hydrolysis of methyl hydrocinnamate by chymotrypsin decreased by 40 % in going from 15 to 25 weight percent of methanol, while LAIDLER AND ETHIER[6] found that the enzymic hydrolysis of adenosine triphosphate was 20 % higher in 40 volume percent methanol than in water. These latter authors found more complicated variations of rate in dioxane–water mixtures. KIMMEL AND SMITH[7] studied the papain-benzoyl-L-arginine amide system in various alcohol–water mixtures. Ethanol had little effect on the maximum rate, while methanol and propanol-2 decreased it.

NELSON AND SCHUBERT[8] studied the enzymic hydrolysis of sucrose at high concentrations of the substrate in water, and in 10 % and 20 % ethanol. In this system the data indicated that the rate is the same function of water concentration whether this is lowered by sucrose or by ethanol.

LAIDLER[5,6,9] has discussed the influence of solvent on the kinetics of enzymic reactions in terms of the effect of dielectric constant on electrostatic interactions, and has shown that the behavior observed in various systems can be reasonably interpreted on this basis. It will appear below that the dependence of the kinetics of the trypsin-catalyzed hydrolysis of BAEE on solvent composition is too complicated to permit any such simple treatment, although it will be of interest to invoke electrostatic interactions in connection with some of the observed phenomena.

All trypsin-catalyzed reactions which have been subjected to kinetic analysis appear to follow the simple MICHAELIS-MENTEN mechanism under usual experimental conditions:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_{2(\mathrm{app})}} E + P \tag{1}$$

As pointed out in earlier papers[10,11] measurements of the over-all rate of product formation by classical (as contrasted with fast-reaction) techniques can only lead to values for the maximum rate, $k_{2(\mathrm{app})}$, and the apparent MICHAELIS-MENTEN constant, $K_{m(\mathrm{app})} = (k_{-1} + k_{2(\mathrm{app})})/k_1$. It should be kept in mind that in cases where the mechanism is more complicated than indicated by eqn. (1), the kinetic constants will include several individual rate constants.

We shall assume in subsequent discussion that $K_{m(\mathrm{app})}$ is the dissociation constant for the enzyme–substrate complex. The only justification for this assumption, aside from convenience, is that the variation of $K_{m(\mathrm{app})}$ with experimental conditions appears to be quite independent of that of $k_{2(\mathrm{app})}$.

EXPERIMENTAL

Materials

Dioxane was purified by a modification of the method of KRAUS AND VINGEE[12]. Commercial "purified dioxane" was stored over NaOH pellets for several days, refluxed over sodium wire for 2–3 h, and then distilled over sodium at atmospheric pressure. A central cut of the distillate was stored over sodium under an atmosphere of nitrogen; exposure to atmospheric oxygen during use was kept to a minimum. Dioxane stored in this way does not contain sufficient sodium to give a flame test, and the pH of a solution in water is identical with that of a similar solution of freshly distilled dioxane. Dioxane concentrations are given in volume percent, a 30% solution being prepared by diluting 3 volumes of dioxane to 10 volumes with water.

BAEE was purchased from Mann Biochemical Co., New York, N.Y., and was used without further purification. M.p. 130° (lit.[3] 129–130°). SMITH AND PARKER[13] have recently reported kinetic anomalies in the hydrolysis of BAEE by papain at 37°, which they attributed to impurities in the substrate which were removed by chromatography. We have observed no such anomalies in the hydrolysis of BAEE by trypsin at 25°. Tris(hydroxymethyl)aminomethane (THAM) was recrystallized from ethanol. Other reagents were of analytical grade.

Crude trypsin, purchased from British Drug Houses, Ltd., was treated[14] as follows. The powdered material was extracted with 0.01 *N* HCl and the trypsin was precipitated with 3% trichloroacetic acid. The precipitate was transferred to a dialysis sac and dissolved by dialysis against 0.01 *N* HCl. The solution was taken to pH 8 and a small amount of precipitate removed by centrifugation. The trichloroacetic acid precipitation and solution in 0.01 *N* HCl were repeated. Stock solutions prepared in this way contained 2.6–3.0 mg N/ml, and had tryptic activities (BAEE substrate) of $3\text{–}4 \cdot 10^{-6}$ moles sec^{-1} mg N^{-1} measured at 25° and pH 8.0 in a solution containing 0.01 *M* phosphate and 0.1 *M* NaCl, with a substrate concentration sufficient to saturate the enzyme. The maximum activity observed was $4.18 \cdot 10^{-6}$ moles/sec/mg N. From the maximum rate of hydrolysis found by GREEN AND NEURATH[15] and the molecular weight (23,800) and N content (14.4%) reported by CUNNINGHAM[16], we can conclude that 99.0% of the nitrogenous material in this sample was trypsin*. Some preparations had as low as 80% of the specific activity of this sample, but were indistinguishable from it so far as relative kinetic behavior was concerned.

Some of the enzyme prepartions were found to have considerable activity towards acetyl-L-tyrosine ethyl ester (ATEE), perhaps in part because of contamination by chymotrypsin. Any such contamination was shown to have negligible effect on the rate of BAEE hydrolysis by experiments in the presence of indole, a good inhibitor for chymotrypsin. Thus in one experiment, 0.01 *M* indole reduced the rate of hydrolysis of ATEE ($2.5 \cdot 10^{-3}$ *M*) by 85% without detectably changing the rate of hydrolysis of BAEE. Indole at a concentration of 0.1 *M* had no effect on the BAEE hydrolysis in 50% dioxane between pH 8 and 11, and in 85% dioxane at pH 8.

Trypsin concentrations were in all cases inferred from the observed activity under assay conditions (25° and pH 8 in aqueous solution containing 0.005 *M* THAM and 0.025 *M* CaCl_2) which we shall hereafter refer to as standard conditions, using the data of GREEN AND NEURATH[15] and CUNNINGHAM[16].

pH Scale

No satisfactory definition of a pH scale in dioxane–water mixtures, in terms of standard buffers, is available. MARSHALL AND GRUNWALD[17] have shown that the glass electrode responds to changes in hydrogen ion activity in the same way as the hydrogen electrode in solutions up to 82 % (by weight) dioxane. FREDERICQ[18] found that a glass electrode and a saturated KCl bridge leading to a reference electrode can be employed to measure pH in 40 % dioxane with the same meaning as the measurement has in water.

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* In arriving at this figure use has been made of our observation that the rate of hydrolysis under the conditions given above is 0.8% lower than under the conditions used by GREEN AND NEURATH[15], namely 25°, pH 8 in a solution containing 0.005 *M* THAM and 0.001 *M* CaCl_2.

We have found the usual pH measuring equipment including a Beckman glass electrode to function conveniently and reproducibly in solutions containing as much as 88 % dioxane, and have adopted the following simple and operationally satisfactory procedure for setting up an arbitrary "pH scale". The pH meter and electrodes were standardized in water using Beckman pH 7 standard buffer, and were then used directly in dioxane–water mixtures to measure the apparent pH. That pH values read in this way are closely related to pH values in water is indicated by the fact that the apparent pH at 25° of a 0.05 *M* THAM–0.05 *M* THAM·HCl solution remained constant at 7.99 ± 0.02 as the dioxane concentration was increased from 0 to 88 %. This behavior would be expected if the pH scale were approximately correct since the ionization of THAM is isoelectric and therefore relatively unaffected by changes in dielectric constant. In contrast, the apparent pH of a 0.05 *M* formic acid–0.05 *M* sodium formate solution increased from 3.72 in water to 6.11 in 80 % dioxane.

Kinetic measurements

The hydrolysis of BAEE at neutral pH is accompanied by the liberation of one proton per molecule hydrolyzed, so that the rate of the reaction was conveniently followed by the method of SCHWERT *et al.*[1]. The reaction solution, usually 10 ml, was immersed in a water bath controlled to 0.05° and was stirred by a magnetic stirrer. Nitrogen gas previously saturated with the solvent being used was led over the solution. At low pH values suitable corrections for incomplete ionization of benzoylarginine were applied to the rates of alkali consumption. These corrections were evaluated from titration curves for benzoylarginine which led to the apparent p*K*′ values listed in Table I. The fact that these values increase less with increasing dioxane concentration than was observed in the case of formic acid is presumably to be attributed to increasing electrostatic interaction between the guanido and carboxyl groups as the dielectric constant is decreased. At high pH values corrections for hydroxide ion catalysis were applied.

TABLE I

APPARENT pK′ VALUES FOR BENZOYLARGININE AT 25° IN AQUEOUS SOLUTIONS CONTAINING 0.025 *M* $CaCl_2$

Percent dioxane	0	50	70	80	85	88
pK′	3.34	4.59	4.87	4.69	4.75	4.57

Titration of benzoylarginine showed that even at pH 11 there was no significant loss of protons from the guanido groups.

Some of the later rate measurements employed a pH-stat of the type developed at the Carlsberg Laboratory[19].

In high concentrations of dioxane at high pH values the curves of alkali consumption *vs* time showed pronounced curvature. Initial rates were estimated graphically by two independent methods. In one method the observed curve was extrapolated to $t = 0$ and the slope estimated directly. In the other the mean rate of alkali consumption over short intervals of time was plotted against the time and extrapolated to $t = 0$; these plots were in most cases very nearly linear. The two methods gave values for the initial rate agreeing on the average to better than 5 %. We estimate the precision of the initial rates to be ± 5 %, except at the higher pH values in 80 to 88 % dioxane.

The initial rates obtained at varying initial substrate concentrations under each constant set of conditions were used to construct EADIE[20] plots, in which the rate is plotted against the ratio of the rate to the initial substrate concentration, for the evaluation of kinetic constants. The "best" straight line through the points in each EADIE plot was determined on the basis of a least squares criterion. Fig. 1 represents an EADIE plot, with y replacing the initial rate and x the substrate concentration.

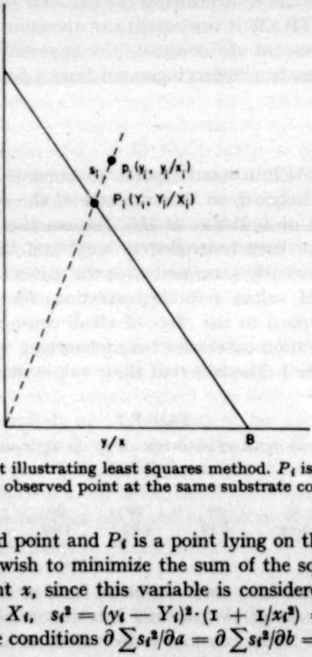

Fig. 1. Schematic EADIE plot illustrating least squares method. P_i is a point on the "true" line, and p_i is the observed point at the same substrate concentration.

Suppose p_i is an observed point and P_i is a point lying on the "true" line AB given by $Y = a + bY/X$. We wish to minimize the sum of the squares of the deviations, s_i, along lines of constant x, since this variable is considered to be essentially free from error. Since $x_i = X_i$, $s_i^2 = (y_i - Y_i)^2 \cdot (1 + 1/x_i^2) = [y_i - ax_i/(x_i - b)]^2 \cdot (1 + 1/x_1^2)$. Applying the conditions $\partial \sum s_i^2/\partial a = \partial \sum s_i^2/\partial b = 0$ gives the equations

$$\sum \left(1 + \frac{1}{x_i^2}\right) \frac{ax_i^2 + bx_iy_i - x_i^2y_i}{(x_i - b)^2} = 0 \qquad (2)$$

$$\sum \left(1 + \frac{1}{x_i^2}\right) \frac{ax_i^2 + bx_iy_i - x_i^2y_i}{(x_i - b)^3} = 0 \qquad (3)$$

In the present application the error in y at small values of x is larger than at large values of x. We have therefore least squared only the y-component of s_i; this amounts to introducing a weighting factor which gives greater weight to the deviations at large x. Eqns. (2) and (3) then become

$$\sum \frac{ax_i^2 + bx_iy_i - x_i^2y_i}{(x_i - b)^2} = 0 \qquad (4)$$

$$\sum \frac{ax_i^2 + bx_iy_i - x_i^2y_i}{(x_i - b)^3} = 0 \qquad (5)$$

These equations are conveniently solved by a method of successive approximations. It can be shown that if b_1 is an approximate value for b, then a better approximation is $b_1 + \Delta b_1$, where

$$\Delta b_1 = \frac{a_1a_4 - a_2a_3}{a_1a_5 - a_2a_4} \qquad (6)$$

with

$$a_1 = \sum \frac{x_i^2}{(x_i - b_1)^2}\,;\; a_2 = \sum \frac{x_i^2}{(x_i - b_1)^3}\,;\; a_3 = \sum \frac{x_iy_i}{x_i - b_1}\,;$$

$$a_4 = \sum \frac{x_iy_i}{(x_i - b_1)^2}\,;\; a_5 = \sum \frac{x_iy_i}{(x_i - b_1)^3} \qquad (7)$$

The value of a corresponding to $b_1 + \Delta b_1$ is

$$a = \frac{a_3a_5 - a_4^2}{a_1a_5 - a_2a_4} \qquad (8)$$

Some typical EADIE plots are given in Fig. 2. The rates, r, are relative rates obtained by dividing the observed rates, in moles of ester hydrolyzed per ml per sec, by the rate observed in water at pH 8.0 with the same enzyme concentration but a substrate concentration large enough to insure saturation of the enzyme. The intercept of an EADIE plot at infinite substrate concentration gives the value of r_{max}, and the slope is equal to $-K_{m(\text{app})}$.

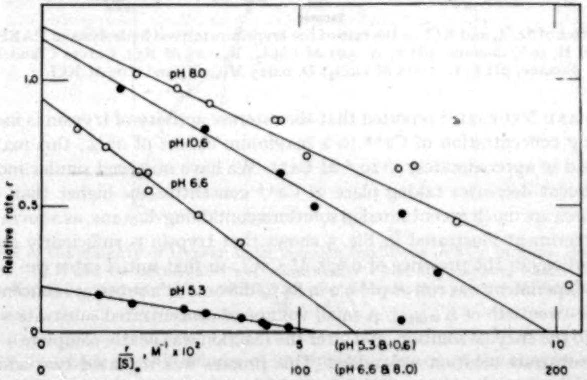

Fig. 2. The trypsin-catalyzed hydrolysis of BAEE in 50% dioxane at 25°. Typical EADIE plots at various values of the pH.

RESULTS AND DISCUSSION

The effect of calcium chloride

Preliminary experiments showed that in 50 % dioxane containing 0.05 M KCl the rate of BAEE hydrolysis decreased during the reaction, while in aqueous medium the rate remained constant until very near to completion. Since the possibility existed that this was due to denaturation of the enzyme and since Ca^{++} has been reported[21, 22] to have a stabilizing influence on trypsin, the effect of $CaCl_2$ was investigated. As seen in Fig. 3, curves A and B, 0.01 M $CaCl_2$ in the absence of KCl is quite effective in decreasing the curvature of the reaction plot. Curves C and D indicate that the presence of KCl counteracts the effect of $CaCl_2$; it is quite unlikely that the difference between curves C and D is due to an increase in $K_{m(\text{app})}$ caused by the KCl, since the substrate concentration employed was approximately 100 times the $K_{m(\text{app})}$ in the absence of KCl.

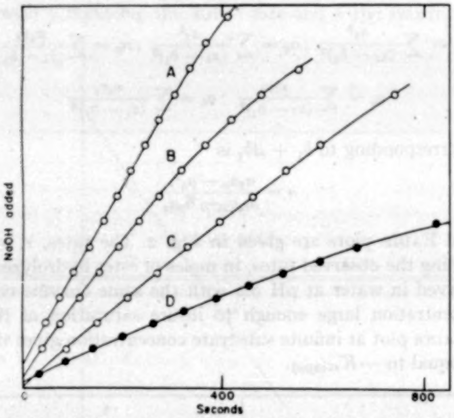

Fig. 3. The effect of $CaCl_2$ and KCl on the rate of the trypsin-catalyzed hydrolysis of BAEE at 25°. Curves A and B, 50% dioxane, pH 8; A, 0.01 M $CaCl_2$; B, 0.05 M KCl. Curves C and D, 70 % dioxane, pH 8; C, 0.025 M $CaCl_2$; D, 0.025 M $CaCl_2$ and 0.05 M KCl.

GREEN AND NEURATH[15] reported that the esterase activity of trypsin is increased by increasing concentration of Ca^{++} to a maximum extent of 28 %, this maximum being reached in approximately $5 \cdot 10^{-4}$ M Ca^{++}. We have observed similar increases, with subsequent decreases taking place at Ca^{++} concentrations higher than 0.1 M; these decreases are much accentuated in solutions containing dioxane, as seen in Fig. 4.

The experiment illustrated in Fig. 5 shows that trypsin is sufficiently stable in dioxane solutions in the presence of 0.025 M $CaCl_2$ so that initial rates can be evaluated. This experiment was run at pH 9.2 in 85 % dioxane at a substrate concentration of about one-twentieth of $K_{m(\text{app})}$. A small volume of concentrated substrate solution was added to the enzyme solution, and after the reaction was nearly complete a second volume of substrate solution was added. This process was repeated two additional times. The approximate constancy of the initial rates indicates that no significant loss of activity took place during a period of 40 min. Thus our usual experimental

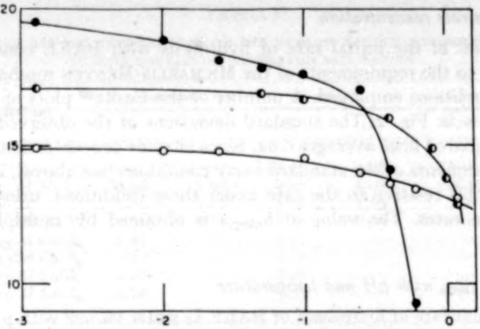

Fig. 4. The effect of CaCl$_2$ on the rate of the trypsin-catalyzed hydrolysis of BAEE at 25°. Open circles, water; half-filled circles, 30% dioxane; filled circles, 50% dioxane.

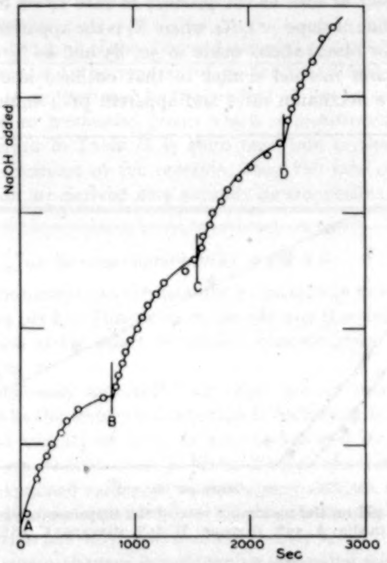

Fig. 5. Test of the stability of trypsin under reaction conditions in 85% dioxane. Substrate was added at A, B, C and D. Initial substrate concentrations and approximate relative initial rates were respectively: A, $1.30 \cdot 10^{-4}$ *M*, (1.00); B, $1.40 \cdot 10^{-4}$ *M*, 1.05; C, $1.51 \cdot 10^{-4}$ *M*, 1.08; D, $1.78 \cdot 10^{-4}$ *M*, 1.18.

procedure, in which a small volume of an aqueous solution of the enzyme was added to the substrate in the appropriate solvent, should be quite adequate. We have not investigated the long term stability of the enzyme in dioxane solutions.

The effect of substrate concentration

The variation of the initial rate of hydrolysis with BAEE concentration was found to adhere to the requirements of the MICHAELIS-MENTEN mechanism under all experimental conditions employed. A number of the EADIE[20] plots obtained in 50 % dioxane are given in Fig. 2. The standard deviations of the observed relative rates from the least squared lines averages 0.02. Since enzyme concentrations were inferred from rate measurements at the standard assay conditions (see above), it is convenient to express all rates relative to the rate under these conditions, using the symbol r for such relative rates. The value of $k_{2(app)}$ is obtained by multiplying r_{max} by 14.8 sec^{-1}.

The variation of r_{max} with pH and temperature

The maximum rate of hydrolysis of BAEE in water varies[2] with pH in a manner consistent with the hypothesis that a group in the catalytic site with an apparent pK' appropriate for an imidazolyl group must be unprotonated for the enzyme to be active. We have found similar behavior in dioxane–water mixtures. According to this hypothesis, a plot of rate *vs* the product of rate times hydrogen ion activity should be a straight line of slope $-1/K_i$, where K_i is the apparent ionization constant. Plots of this type for observations made in 50, 85 and 88 % dioxane are given in Fig. 6. A least squares method similar to that outlined above was employed to calculate the relative maximum rates and apparent pK'_i values, determined under conditions of enzyme saturation, which are given in Table II, together with apparent heats of ionization and apparent heats of activation. Not only are the pK'_i values and apparent heats of ionization in Table II entirely reasonable for an imidazolyl group, but the fact that they are so little affected by large changes in dielectric constant indicates that the ionization involved is probably isoelectric.

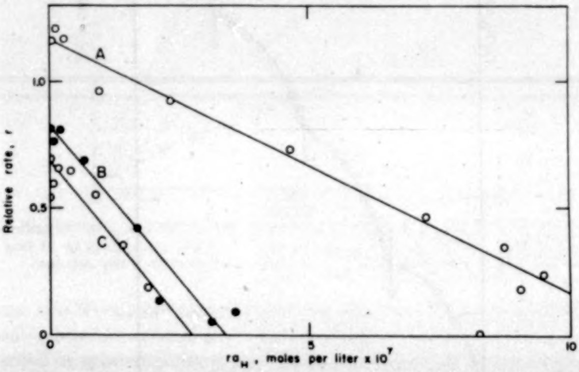

Fig. 6. The influence of pH on the maximum rate of the trypsin-catalyzed hydrolysis of BAEE in various media. A, 50% dioxane; B, 85% dioxane; C, 88% dioxane.

The pK'_i values at 25° and 35° given in the second line of Table II differ slightly from those reported by GUTFREUND[2], and the apparent heat of ionization is approx-

TABLE II

THE EFFECT OF pH AND TEMPERATURE ON THE RATE OF TRYPTIC HYDROLYSIS OF BAEE UNDER CONDITIONS OF ENZYME SATURATION

Percent dioxane	*Added salt*	*Maximum relative rate*		*pK'$_i$*		*Apparent heat of ionization, cal/mole*	*Apparent heat of activation, cal/mole*
		25°	*35°*	*25°*	*35°*		
0	0	0.97	1.67	6.06	5.88	7600	9300
0	0.05 *M* KCl	0.99	1.68	6.22	6.01	8800	9100
50	0	1.19	2.04	5.80	5.63	7200	9200
50	0.05 *M* KCl	1.04	1.67	6.19	6.01	7600	8100
0	0.025 *M* $CaCl_2$	1.01	—	6.02	—	—	—
50	0.025 *M* $CaCl_2$	1.17	—	6.00	—	—	—
85	0.025 *M* $CaCl_2$	0.82	—	6.38	—	—	—
88	0.025 *M* $CaCl_2$	0.68	—	6.40	—	—	—

imately 2000 cal/mole larger and the apparent heat of activation 2000 cal/mole smaller than the quantities found by GUTFREUND. Some of these differences appear to be due to the fact that the ionic strength in our experiments was less than half that used by GUTFREUND.

It should be noted that all of the observed parameters pertaining to the ionization of the active site can also be accounted for on the hypothesis that the rate of the rate-controlling step is proportional to the product of the activity of hydroxide ion times the fraction of an imidazolyl group which is protonated. The interpretation of the heat values given in Table II is quite uncertain in the absence of detailed knowledge of the mechanism of the reaction. Thus the rate constants from which the heats of activation are derived may actually be composites of several individual rate constants.

The dependence of r_{max} on dioxane concentration at pH 8.0

At all dioxane concentrations the rate has its maximum value over a range of pH values which includes pH 8.0. This value of the pH was therefore selected for a more extensive investigation of the effect of dioxane concentration, the results of which are summarized in Fig. 7.

As has been previously reported[3,6] for other solvent mixtures, the maximum rate at first increases as the water concentration is decreased. In the present case, the flat maximum in the vicinity of 50 % dioxane is followed by a decrease at higher dioxane concentrations, though even in 88 % dioxane the rate is still 68 % of its value in water. As explained earlier, it would be very difficult to carry the measurements to still higher dioxane concentrations; this is unfortunate, since in 88 % dioxane the water concentration has only been reduced to 6.65 *M*.

Such relatively minor changes in rate can be accounted for in a variety of ways. Thus, it might be argued that the changes in the dielectric constant of the solvent are sufficient to alter electrostatic interactions within the enzyme molecule and thus to produce slight changes in some critical spacing at the catalytic site. It is, of course, quite impossible at present to prove or disprove suggestions of this sort.

The maximum rate under a given set of conditions involves the interaction of an enzyme derivative with either free or bound water, or possibly with hydroxide ions. If we neglect the latter possibility, and secondary effects such as the one mentioned

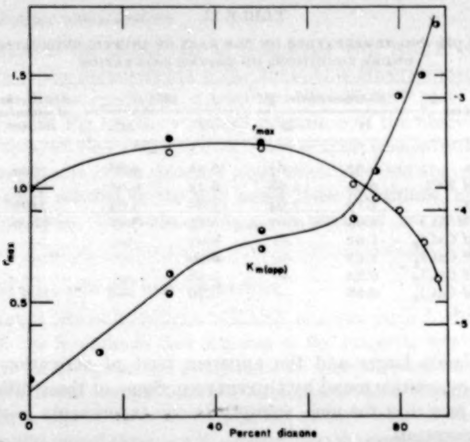

Fig. 7. The effect of dioxane concentration on the maximum rate and $K_{m(app)}$ of the tryptic hydrolysis of BAEE at 25° and pH 8.0 in the presence of 0.025 *M* $CaCl_2$. Maximum rates: ○ 0.005 *M* THAM; ●, no THAM. $K_{m(app)}$: ◑, 0.005 THAM; ⊖, no THAM.

in the preceding paragraph, we should expect the rate to be essentially independent of dielectric constant and mainly dependent on water concentration. In the case that the overall reaction follows simple MICHAELIS-MENTEN kinetics and water enters into a bimolecular reaction with the enzyme–substrate complex, the dependence of r_{max} on water concentration should follow an equation of the form

$$r_{max} = k_{2(app)}\,[E]_0\,[H_2O] \tag{9}$$

It is evident from Fig. 7 that this equation does not hold. If, on the other hand, the reaction adheres to a 3-step mechanism[10]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES' \overset{k_2}{\rightleftarrows} ES'' + P' \tag{10}$$

$$ES'' + H_2O \overset{k_3}{\rightleftarrows} E + P'' \tag{11}$$

"initial" rates observed after the formation of the steady-state concentration of *ES''* should follow the expression

$$r_{max} = \frac{k_{2(app)}\,[E]_0\,[S]_0}{K_{m(app)} + [S]_0} \tag{12}$$

with

$$k_{2(app)} = k_2\,\frac{[H_2O]}{\dfrac{k_2}{k_3} + [H_2O]} \tag{13}$$

and

$$K_{m(app)} = \frac{k_{-1} + k_2}{k_1} \cdot \frac{[H_2O]}{\dfrac{k_2}{k_3} + [H_2O]} \tag{14}$$

Consideration must also be given to the possibility that water must be bound by the enzyme before it can enter into the hydrolysis. For example, if the scheme

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \tag{15}$$

$$E(H_2O) + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E(H_2O)S \tag{16}$$

$$ES + H_2O \rightleftarrows E(H_2O)S \tag{17}$$

$$E(H_2O)S \xrightarrow{k_2} E + P \tag{18}$$

applies, then the steady-state approximation gives eqn. (12) with

$$k_{2(\mathrm{app})} = k_2 \frac{[H_2O]}{K_h + [H_2O]} \tag{19}$$

$$K_{m(\mathrm{app})} = \frac{k_{-1} + k_2 \dfrac{[H_2O]}{K_h + [H_2O]}}{k_1} \tag{20}$$

on the assumption that the binding of water attains an equilibrium which is independent of the binding of substrate. K_h is the dissociation constant of the enzyme–water complex. We thus have at least two cases giving a variation of maximum rate with $[H_2O]$ of the form

$$k_{2(\mathrm{app})} = k' \frac{[H_2O]}{k'' + [H_2O]} \tag{21}$$

according to which an EADIE plot of $r_{\max}$ *vs* $r_{\max}/H_2O$ should be a straight line.

It is evident that the 3-step mechanism with bound water required in the last step also leads to eqn. (12), but with

$$k_{2(\mathrm{app})} = \frac{k_2 k_3}{k_2 + k_3 \dfrac{[H_2O]}{K_h + [H_2O]}} \cdot \frac{[H_2O]}{K_h + [H_2O]} \tag{22}$$

and

$$K_{m(\mathrm{app})} = K_m \frac{k_3}{k_2 + k_3 \dfrac{[H_2O]}{K_h + [H_2O]}} \cdot \frac{[H_2O]}{K_h + [H_2O]} \tag{23}$$

According to eqn. (22) the dependence of $k_{2(\mathrm{app})}$ on $[H_2O]$ will be of the form of eqn. (21) if the reaction is rate-limited at the final step, whereas if the reaction is rate-limited at the second step $k_{2(\mathrm{app})}$ will be independent of $[H_2O]$. (The dependence of $K_{m(\mathrm{app})}$ on $[H_2O]$ is discussed in a later section.)

Above 50% dioxane (for an admittedly short range of values of $r_{\max}/[H_2O]$) the behavior is consistent with eqn. (21). The decrease in rate at high water concentrations is evidently not specific with respect to dioxane, having been previously observed[3] for ethanol–water mixtures. We have observed a similar effect with water–dimethylsulfoxide mixtures (see below). The non-specific nature of this effect suggests that it may result from an inhibitory effect of water, for example in the sense of substrate inhibition. If we assume the mechanism of eqns. (15) to (18), and add the assumption that the species $E(H_2O)_2$ (or $E(H_2O)_2S$) cannot react, then

$$k_{2(\mathrm{app})}\left(1 + \frac{[H_2O]}{K_I}\right) = k_2 - K_h \frac{k_{2(\mathrm{app})}}{[H_2O]} \tag{24}$$

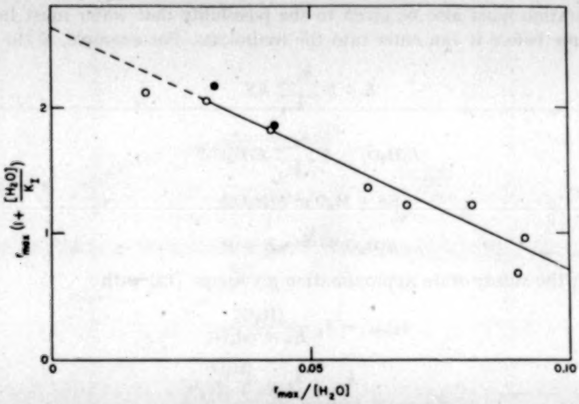

Fig. 8. The variation with water concentration of the maximum rate of the tryptic hydrolysis of BAEE, as interpreted in terms of specifically bound water and inhibition by water (see text).

where K_I is the equilibrium constant for the dissociation of the inhibiting molecule of water. It is found that the value $K_I = 50\,M$ leads to adherence to eqn. (24) within experimental error, as shown in Fig. 8. The slope of the plot gives the value $K_h = 19\,M$ and a hypothetical maximum rate 2.6 times that observed in water.

Other types of mechanism could be invoked to account for the observed variation of r_{max} with solvent composition. The above treatment is only intended to show that the experimental data can be reconciled with reasonable kinetic schemes; it cannot be proposed as a unique interpretation.

KOSHLAND AND HERR[23] have shown that water is a much more effective acceptor for the phosphate split from adenosine triphosphate by myosin than is methanol. This finding is consistent with the hypothesis that the water entering into an enzymic hydrolysis is specifically bound by the enzyme before it reacts.

The dependence of $K_{m(app)}$ *on dioxane concentration at pH 8*

The strong influence of dioxane concentration on $K_{m(app)}$ at pH 8.0 is shown in Fig. 7. Between 0* and 88 % dioxane an increase by a factor of 1300 is observed. The value in water, $3.4 \cdot 10^{-6}\,M$, is approximately one-third that reported by BERNHARD[24] for experimental conditions differing in several respects from those employed here.

The mechanisms discussed above in connection with the variation of r_{max} with dioxane concentration predict small decreases of $K_{m(app)}$ with increasing dioxane concentration. It is usually assumed that an important part of the energy of binding of BAEE to trypsin is electrostatic in origin, the positive guanidyl group interacting with a negative group on the enzyme. It would then be expected that this interaction would increase as the dielectric constant is decreased and that $K_{m(app)}$ would therefore decrease.

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* In the determination[24] of $K_{m(app)}$ at low dioxane concentrations it is necessary to use large volumes of reaction solution in order to have rates of alkali consumption large enough for convenient measurement.

It is nevertheless conceivable that the large increase in $K_{m(\mathrm{app})}$ results from increased electrostatic repulsion. If the positive charge on the substrate comes very close to a negative charge on the enzyme, the interaction of these charges may be much less affected by changes in the dielectric constant of the medium than is the interaction of the positive charge of the substrate with the net positive charge which trypsin carries at pH 8. Application of the LINDERSTRØM-LANG[25] treatment based on the limiting DEBYE-HÜCKEL theory, which is of course a very rough approximation in media of low dielectric constant, gives for the effective charge on trypsin reasonable values in the range + 5 to + 12 depending on the solvent composition. In these calculations the radius of the trypsin molecule was taken as 19 Å, and $CaCl_2$ was necessarily assumed to be a strong electrolyte. Values of d log $K_{m(\mathrm{app})}$/d $(1/D)$, where D is the dielectric constant[26], were read from the curve in Fig. 9.

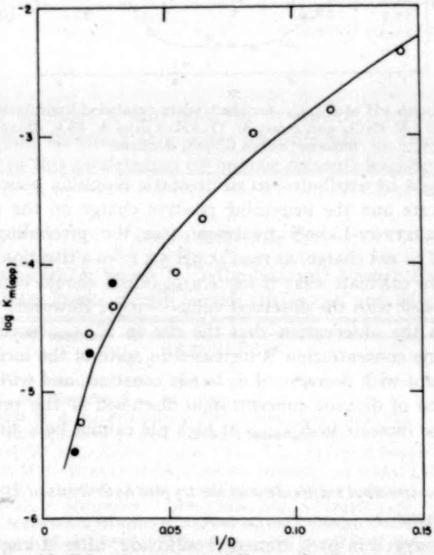

Fig. 9. The variation with dielectric constant of $K_{m(\mathrm{app})}$ for the trypsin-catalyzed hydrolysis of BAEE at 25° and pH 8 in the presence of 0.025 M $CaCl_2$. Open circles, 0.005 M THAM; filled circles, no THAM.

The possibility cannot be excluded that the change in $K_{m(\mathrm{app})}$ with solvent composition is primarily the result of configurational changes, which would not have to be very pronounced, and that any direct dielectric constant effect is of relatively minor significance.

The variation of $K_{m(app)}$ *with pH*

At a fixed dioxane concentration, $K_{m(\mathrm{app})}$ increases at pH values both below and above the pH range in which $r_{\max}$ has its maximum value. This behavior is shown in Fig. 10. It is difficult to devise consistent explanations for these effects. Thus the

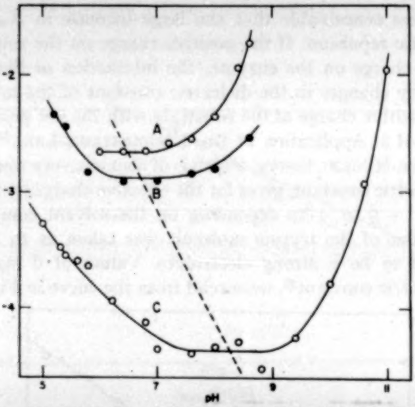

Fig. 10. The variation with pH of $K_{m(app)}$ for the trypsin-catalyzed hydrolysis of BAEE at 25° in the presence of 0.025 M $CaCl_2$ and 0.005 M THAM. Curve A, 88% dioxane; curve B, 85% dioxane; curve C, 50% dioxane.

low pH increase might be attributed to electrostatic repulsion between the guanido group of the substrate and the increasing positive charge on the enzyme. On the basis of the LINDERSTRØM-LANG[25] treatment (see the preceeding section), with $dZ/dpH = -5.0$ (Z = net charge) as read at pH 5.5 from a titration curve of trypsin in 50% dioxane, we calculate that $d \log K_{m(app)}/dpH$ should equal -0.60. This agrees fortuitously well with the observed value -0.57. However, this explanation is inconsistent with the observation that the rise in $K_{m(app)}$ occurs at lower pH values as the dioxane concentration is increased in spite of the increase of the pK′ of the carboxyl groups with decrease of dielectric constant, and with the increase of $K_{m(app)}$ with increase of dioxane concentration discussed in the preceding section. It is evident that the increase in $K_{m(app)}$ at high pH cannot be a direct electrostatic effect.

Stability of trypsin in dimethyl sulfoxide and the tryptic hydrolysis of BAEE in dimethyl sulfoxide–water solutions

Trypsin was dissolved in 97% dimethyl sulfoxide; after storage at 12.5° or 5° for 14 h, the solution was diluted with 10 volumes of water and the activity toward BAEE determined. When the dimethyl sulfoxide solution contained 0.05 M $CaCl_2$, recovery of activity amounted to 93%; in the absence of $CaCl_2$, recovery after storage at 12.5° amounted to 79%, but only 45% after storage at 5°, at which temperature the solution was frozen.

A few determinations of the initial rate of BAEE hydrolysis in dimethyl sulfoxide solutions at 25° are summarized in Table III. These determinations were all made with $5 \cdot 10^{-3}$ M BAEE. The pH scale was defined operationally in the same manner as for dioxane–water mixtures. Deviations from zero order kinetics were evident, even at low dimethyl sulfoxide concentrations. It is not known whether this is a manifestation of large values of $K_{m(app)}$ or of other effects.

It is seen from the data in Table III that an acceleration in rate similar to that

TABLE III

RELATIVE INITIAL RATE OF HYDROLYSIS OF BAEE BY TRYPSIN IN DIMETHYL SULFOXIDE SOLUTIONS AT 25°

Rates are relative to the rate in water at 25°, pH 8.0, in the presence of 0.025 *M* CaCl, 0.005 *M* THAM. Initial substrate concentration $5 \cdot 10^{-3}$ *M*.

Dimethyl sulfoxide concn., volume %	*pH*							
	7.5	*8.0*	*8.4*	*8.5*	*8.7*	*9.0*	*9.2*	*9.5*
0		1.00				1.01		
10						1.05		
20	1.06	1.08		1.09		1.11		
30		1.21		1.30				
40		1.24		1.29				
50	1.06	1.17		1.27		1.27		
55						1.24		
60	0.79	0.88	0.98		1.08	1.06	0.85	0.59
65					0.67	0.78		
70						0.61		

found in dioxane solutions is also observed in dimethyl sulfoxide solutions, the maximum rate again occurring at a water concentration of about 50%. The non-specific character of this acceleration by organic solvents lends support to the view that a weak inhibition by water occurs in the trypsin-BAEE system.

ACKNOWLEDGEMENTS

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ACTIVE AND INACTIVE MODIFICATIONS OF TRYPSIN INDUCED BY ACETYLATION*

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SUMMARY

The acetylation of trypsin with acetic anhydride results in a heterogeneous mixture of acetylated derivatives. That fraction which is most fully acetylated (85–90 %) is enzymically inactive, whereas the partially acetylated (about 50 %) fraction is fully as active as unmodified trypsin. These two forms of trypsin may be further differentiated on the basis of their solubility properties, chromatographic behavior on DEAE-cellulose, and optical rotation. The implication of these results with respect to the structural requirements for enzymic activity is discussed.

INTRODUCTION

Chemical modification of proteins[1,2] has, in many instances, proved to be a useful tool in delineating the specific groups responsible for their biological activity. In the case of trypsin, FRAENKEL-CONRAT *et al.*[3] and NORD and his associates[4,5] have reported that the acetylation of amino groups does not interfere with the catalytic function of this enzyme. In this laboratory, however, it has been consistently observed that the acetylation of trypsin, employing acetic anhydride under the same conditions prescribed by these authors[3–5], does result in a noticeable loss of activity. In an attempt to resolve these discrepancies, the acetylation of trypsin has now been subjected to closer scrutiny. The evidence to be presented here shows that the reaction of trypsin with acetic anhydride leads to a heterogeneous mixture containing active and inactive species of modified protein which can be further differentiated on the basis of certain physical and chemical properties.

EXPERIMENTAL

Trypsin was acetylated with acetic anhydride in the manner originally described by FRAENKEL-CONRAT *et al.*[3] and likewise adopted by NORD's group[4,5]**. Since this paper deals with conflicting results obtained in this laboratory and elsewhere, the experimental conditions assume considerable importance and will be described in detail.

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[*] Paper No. 4076, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul, Minn.

[**] The specificity of this procedure for the acetylation of the amino groups of trypsin has been established by URAKI *et al.*[6] using labelled acetic anhydride. Although O-acetyl groups are also introduced, these are unstable under the alkaline conditions employed in the enzymic assay.

An aqueous solution of 200 mg of twice crystallized salt-free trypsin (Worthington, lot No. 641) dissolved in 40 ml of ice-cold, half-saturated sodium acetate was treated with 0.5 ml acetic anhydride. The latter was added intermittently over a period of 1 h with the enzyme solution immersed in an ice bath and agitated with a magnetic stirrer. The solution was then exhaustively dialyzed at 4° against 0.001 *N* HCl (pH 3) and lyophilized. This preparation will be referred to as "1x-acetyltrypsin".

Where a second acetylation was desired, lyophilization was omitted, and sufficient solid sodium acetate was added to the dialyzed solution (volume about 100 ml) to bring it to half-saturation followed by the intermittent addition of 0.8 ml acetic anhydride over a period of 90 min in the same manner as above. Dialysis against 0.001 *N* HCl and lyophilization gave a preparation designated as "2x-acetyltrypsin".

During dialysis at pH 3, a considerable amount of protein precipitated. Since this insoluble fraction was subsequently found to have very little activity, preparations of acetyltrypsin were also made in which this insoluble protein was removed by centrifugation after each dialysis stage of the preparation. This insoluble fraction could be redissolved at pH 10 for purposes of analysis.

The treatment of trypsin or acetyltrypsin preparations with calcium ions involved the dispersion of the sample in 0.005 *M* borate buffer, pH 8.0, containing 0.05 *M* $CaCl_2$, to give a concentration of 5 mg of protein/ml of buffer. Under these conditions, an inactive fraction of the protein remained insoluble and could be separated by centrifugation. This fraction could be subsequently solubilized by adjusting an aqueous suspension of the precipitate to pH 8.0.

The enzymic activity of the various fractions was determined on benzoyl L-arginine ethyl ester (BAEE) using the spectrophotometric procedure of SCHWERT AND TAKENAKA[7]. 0.05 *M* $CaCl_2$ was incorporated into the substrate solution in order to provide the activating influence of calcium ions[8,9] to systems which had not been previously fractionated with $CaCl_2$. One activity unit is arbitrarily defined as an absorbance change of 1.0 per min at 37°, and the specific activity as the number of activity units per mg protein. The protein concentration (mg/ml) was obtained by multiplying the absorbance of the protein solution at 280 mμ by a factor of 0.6[10], or by multiplying the N content, determined according to LANNI *et al.*[11], by 6.25. A mol. wt. of 24,000 was assumed for converting protein into moles of trypsin or acetyltrypsin[12]. The extent of acetylation was estimated by comparing the number of free amino groups present in the initial and final materials according to the ninhydrin method of TROLL AND CANNAN[13]. The applicability of the ninhydrin reaction for the evaluation of the amino groups of trypsin and its acetylated derivatives has been pointed out by NORD *et al.*[14] and PECHÉRE AND NEURATH[15].

Various preparations or fractions of acetyl-trypsin were examined chromatographically on diethyl-aminoethyl (DEAE)-cellulose in essentially the same manner as described elsewhere[16], namely stepwise elution with solutions of increasing ionic strength in the following sequence: 0.005 *M* borate buffer, pH 8.0; 0.01 *M* borate–0.02 *M* NaCl, pH 8.0; 0.02 *M* phosphate–0.10 *M* NaCl, pH 6.8; and 1% NaOH. Eluates obtained with each of these solutions will be referred to as fractions I, II, III, and IV respectively. Fractions from the column were examined for protein by measuring absorbance at 280 mμ and for enzymic activity on BAEE. Column fractions could not be reliably analyzed with ninhydrin by the method of TROLL AND CANNAN[13] since the level of free amino groups in the protein solutions obtained directly from the column were far below the sensitivity of this method. All samples were adjusted to pH 8.0 or dialyzed against 0.005 *M* borate buffer, pH 8.0, prior to their application to the column. Suspensions or precipitates which were not initially completely soluble at pH 8.0 were first dissolved in alkaline solution (pH not exceeding 10) and then readjusted to pH 8.0. The same column, after re-equilibration with the starting buffer, was used for all the analyses reported here. All chromatographic runs were made in a cold room maintained at a temperature of 4–7°.

RESULTS

In Table I are recorded the activity and amino groups of various preparations or fractions obtained by the acetylation of commercial trypsin (preparation 1). If the insoluble precipitate which formed during the dialysis of the acetylated protein was not removed after the first acetylation, preparation 3 was obtained which had 81 % of its amino groups blocked and had lost about 37 % of its original activity. A second acetylation, without removal of insoluble protein, blocked additional amino groups, but at the expense of a 50 % loss in activity (preparation 4).

If, on the other hand, the insoluble precipitate was removed after dialysis, preparations 5-S and 6-S were obtained after the first and second acetylations

TABLE I

AMINO GROUPS AND ACTIVITY OF PREPARATIONS OF TRYPSIN AND ACETYLATED TRYPSIN

Preparation	*Sub-fractions*	*Designation*	*Protein distribution %*	*Amino groups*		*Specific activity*
				Moles/mole protein	*% blocked*	
Trypsin	None	1	—	12.5	0	21.7
Ca-treated trypsin	Supernate	2-S	65	12.3	0	35.0
	Precipitate	2-P	35	12.6	0	0
1x acetyltrypsin	None[*]	3	—	2.4	81	13.7
2x acetyltrypsin	None[*]	4	—	1.5	88	10.5
1x acetyltrypsin	Suspension after dialysis	5	100	2.6	79	14.0
	Supernate	5-S	56	4.9	61	23.8
	Precipitate	5-P	44	1.9	85	1.5
2x acetyltrypsin[**]	Suspension after dialysis	6	100	3.4	73	19.2
	Supernate	6-S	86	4.2	66	20.8
	Precipitate	6-P	14	1.1	91	1.2
Ca-treated 2x acetyltrypsin[***]	Supernate	7-S	55	6.0	52	33.4
	Precipitate	7-P	45	1.9	85	1.3
1x acetyltrypsin[§]	None	8		3.4	73	28.3
2x acetyltrypsin[§]	Suspension after dialysis	9	100	2.4	81	21.7
	Supernate	9-S	75	3.6	71	28.0
	Precipitate	9-P	25	1.1	91	1.3

[*] Precipitate formed during dialysis was not removed.

[**] Only the supernate from the 1x-acetyltrypsin (preparation 5-S) was acetylated.

[***] The lyophilized supernate from 2x-acetyltrypsin (preparation 6-S) was treated with calcium as described in the text.

[§] The lyophilized supernate from Ca-treated trypsin (preparation 2-S) was acetylated. No precipitation was observed during dialysis after the first acetylation.

respectively. The activities of both of these preparations were quite comparable to the activity of the original trypsin*. This enrichment of activity was evidently due to the removal of a considerable amount of the inert protein precipitating out during dialysis (preparations 4-P and 6-P). This inert protein was further characterized by its high degree of acetylation, 85–90 %, compared to the soluble, active fractions which were acetylated to the extent of only 60–65 %.

Treatment of 2x-acetyltrypsin (preparation 6-S) with borate buffer containing calcium ions resulted in the precipitation of an additional amount of inactive, highly acetylated protein (preparation 7-P). The soluble supernatant solution (preparation 7-S) now had a specific activity of 33.4, far in excess of that of the original trypsin (21.7), and only about 50 % of its amino groups were acetylated.

Since the activity of the original trypsin preparation was less than that of its acetylated derivative which had been treated with calcium ions, it was thought that either calcium was acting as an activator or that the trypsin preparation employed in these studies was relatively impure. The first possibility was considered unlikely for the following reasons: (a) the magnitude of the increased activity was much greater than the 25 % usually attributed to calcium[17], (b) the removal of calcium ions from preparation 7-S by prolonged dialysis did not reduce its specific activity and (c) all assays were conducted in the presence of 0.05 *M* $CaCl_2$ (see experimental). On the other hand, treatment of trypsin with calcium under the same conditions resulted in the formation of an insoluble, inactive fraction (preparation 2-P), which constituted about one-third of the total protein, and a soluble fraction (preparation 2-S) which was about 1.5 times as active as the commercial enzyme preparation. The activity of this soluble fraction was now comparable to the activity of the calcium-treated acetyltrypsin referred to earlier.

The acetylation of preparation 2-S, the active fraction of calcium-treated trypsin, produced little or no precipitable protein during dialysis after the first acetylation (preparation 8). After the second acetylation, however, 25 % of the protein became insoluble (preparation 9-P). As before, this insoluble fraction was highly acetylated, about 90 %, and essentially devoid of activity. The supernatant (fraction 9-S) was only 71 % acetylated and had an activity which was somewhat less than that of the starting enzyme preparation 2-S.

In contrast to solubility behavior, which served a useful but obviously limited means of distinguishing the various fractions of acetyltrypsin, chromatography on DEAE-cellulose permitted a more detailed examination of the composition of these protein fractions. The various chromatographic patterns are shown in Fig. 1–3, and Table II provides additional data regarding the distribution and recovery of the protein and activity in these chromatographic experiments.

Fig. 1 serves to compare two preparations of 2x-acetyl-trypsin, one (preparation 4) in which the insoluble protein which precipitates out during dialysis was not removed, and the other (preparation 6-S) in which this fraction was eliminated. The principal difference between these two patterns is the reduction in the size of fraction IV as a result of removing this insoluble, highly acetylated protein. As a consequence of this redistribution of protein, the active component in fraction III is proportionately greater in preparation 6-S. Of the three components in fraction III, the first

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* It is this observation, perhaps, which may have led other investigators[2–5] to conclude that acetylation does not seriously impair the activity of trypsin.

TABLE II

PROTEIN DISTRIBUTION AND ACTIVITY DATA OF CHROMATOGRAPHIC EXPERIMENTS

Preparation*	*Original sample*		*Column fractions*						
			% protein of the original					*Activity in fraction III*	
	Protein mg	*Specific activity*	*I*	*II*	*III*	*IV*	*Total recovery*	*Spec.***	*Total % recovered*
4 (Fig. 1A)	8.9	11	4	22	35	50	111	31	83
6-S (Fig. 1B)	7.9	21	0	2	56	32	90	34	84
7-S (Fig. 2A)	7.7	33	0	4	83	16	103	36	86
7-P (Fig. 2B)	7.0	1	1	9	21	63	94	6	94
8 (Fig. 3A)	5.8	27	0	7	67	22	96	33	84
9 (Fig. 3B)	7.2	21	0	11	58	40	109	32	77

* See Table I for description of the various preparations.
** Based on activity and protein content of tube corresponding to the active peak.

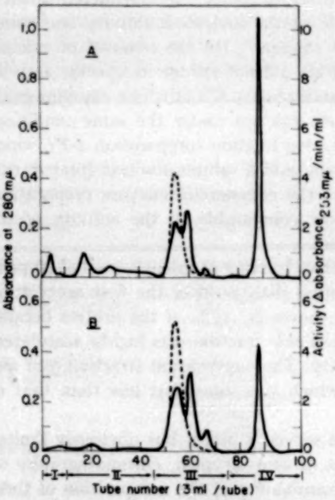

Fig. 1. Chromatographic behavior of 2x-acetyltrypsin showing the effect of removing protein which precipitates out during dialysis. Patterns A and B were obtained with preparations 4 and 6-S respectively described in Table I. Distribution of protein (based on absorbance at 280 mμ) is shown in solid curve, and activity is shown in dashed curve. Changes in elution schedule are indicated by roman numerals below the abcissae. See experimental section for further explanation.

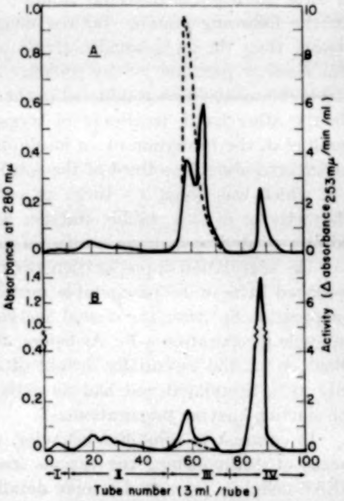

Fig. 2. Chromatographic behavior of fractions of 2x-acetyltrypsin treated with calcium. Patterns A and B were obtained with preparations 7-S and 7-P respectively described in Table I. See caption to Fig. 1 for further explanation of figure.

peak to emerge appears to contain most of the activity which, in terms of specific activity, is comparable to that of the calcium treated trypsin described earlier*.

The effect of treating preparation 6-S with calcium is shown in Fig. 2A where a further diminution in fraction IV and a proportionate increase in fraction III may be noted. That the protein precipitated by calcium is in fact fraction IV is evident from Fig. 2B. The small residual activity associated with the calcium-precipitated protein is evidently due to contamination with fraction III.

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* Unmodified trypsin is not absorbed by DEAE-cellulose under the conditions employed here.

The chromatographic experiments depicted in Fig. 3 were designed to answer the question as to whether the inert protein, as represented by fraction IV, was derived from contaminants pre-existent in the trypsin or arose as a consequence of the acetylation of the active component itself. The starting material in this case was trypsin which had been pre-treated with calcium to remove inactive impurities (preparation 2-S). This preparation was judged as being essentially homogeneous since a single peak was obtained when it was chromatographed on carboxymethylcellulose*. Fig. 3A depicts the pattern obtained after the first acetylation. About 20 % of the protein was converted to inactive protein as represented by fraction IV. After the second acetylation, Fig. 3B, this fraction had increased to 40 %. This progressive increase in fraction IV occurred largely at the expense of fraction III which contains the active component.

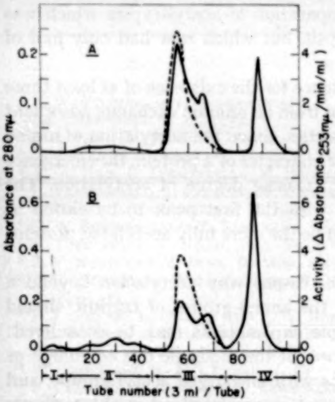

Fig. 3. Chromatographic behavior of acetyltrypsin prepared from calcium treated trypsin. Pattern A was obtained with 1x-acetyltrypsin and B with 2x-acetyltrypsin (preparations 8 and 9 respectively as described in Table I). See caption to Fig. 1 for further explanation of figure.

TABLE III

OPTICAL ROTATION OF ACETYLTRYPSIN AND TRYPSIN PREPARATIONS

*Preparation**	$[\alpha]_{25}^{D}$
6	— 64.9
7-S	— 40.0
7-P	— 80.0
Trypsin	— 45.4
Trypsin in 8 *M* urea	—124.8

* See Table I for description of preparations. All preparations were dialyzed, lyophilized, and redissolved in 0.1 *M* borate buffer, pH 8.0, prior to measurement. Final concentration of protein, 0.5 %. Length of polarimeter cell, 10 cm.

Optical rotations were measured on buffered solutions (pH 8.0) of 2x-acetyltrypsin (preparation 6) and the soluble and insoluble fractions derived therefrom by treatment with calcium ions (preparations 7-S and 7-P respectively). These data are recorded in Table III. The preparation of 2x-acetyltrypsin was more levorotatory than trypsin itself, and no doubt reflects the contribution of the Ca-soluble and Ca-insoluble fractions. The rotation of the Ca-insoluble fraction was twice that of the Ca-soluble fraction, the latter agreeing within experimental error with the value for trypsin. The rotation of urea-denatured trypsin is shown for comparison.

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* Unpublished experiments.

DISCUSSION

The most obvious conclusion to be drawn from this study is that the acetylation of trypsin, performed under the conditions described by FRAENKEL-CONRAT *et al.*[3], results in a heterogeneous mixture of proteins. A partial resolution of this mixture may be achieved by virtue of the fact that the most highly acetylated species are insoluble at pH 3 in the absence of salt, conditions which obtain during the dialysis of the acetylated protein solution. This insoluble fraction, which had 85–90 % of its amino groups blocked, was virtually devoid of activity. On the other hand, the protein which remained soluble under these conditions had only 60–65 % of its amino groups blocked by acetylation. This fraction was fully as active as the starting trypsin preparation but only two-thirds as active as a preparation of trypsin which had been purified by treatment with calcium.

Chromatographic experiments revealed that the almost fully acetylated protein corresponded to a fraction which required 1 % NaOH for its elution from DEAE-cellulose. This fraction was still present to considerable extent in the soluble supernatant, but could be further reduced by subsequent treatment with 0.05 *M* $CaCl_2$. By this means, it was possible to obtain a preparation of acetyltrypsin which was virtually as active as the calcium purified trypsin but which now had only half of its amino groups blocked.

The chromatographic patterns provide evidence for the existence of at least three to four major components whose order of elution from an anionic exchange adsorbent is most likely determined by their anionic properties. Since the acetylation of amino groups would be expected to enhance the anionic character of a protein, the emergence of each component would occur in order of increasing degree of acetylation. The active component was in all cases associated with the first peak to be eluted as fraction III, and the peaks which followed would be the more fully acetylated proteins which were inactive.

It becomes pertinent to inquire as to the reasons why acetylation beyond a critical limit, which seems to be about half of the amino groups of trypsin, should lead to a loss in enzymic activity. Two possible explanations may be considered: (a) Changes in the secondary or tertiary structure of the molecule due to charge or steric effects induced by replacing amino groups with uncharged acetyl groups, and (b) the blocking of less reactable amino groups which are involved in the catalytic function of the enzyme. Evidence in support of (a) is derived from the observation that the specific rotation of the highly acetylated, inactive derivative of trypsin is twice that of the partially acetylated but active derivative which is similar to native trypsin. It has become increasingly apparent from recent studies on the chemical modification of proteins that pronounced changes in physical properties, indicative of denaturation, may be the principal cause for losses in biological activity[18–22]. Although sufficient evidence to exclude (b) is not available from these studies, it is pertinent to recall an analogous situation with such –SH enzymes as urease[23] and aldolase[24] where a decrease in catalytic activity does not occur until the less reactive –SH groups have become blocked. Here again the evidence points to secondary changes in the structure caused by the blocking agent, as being primarily responsible for this loss in activity[24].

The observations recorded here may have a direct bearing on the report by

Nord *et al.*[14] that acetyltrypsin retains its activity while undergoing extensive self-digestion. If the inert protein resulting from "over-acetylation" does in fact represent a form of denatured protein, it is possible that the latter is more susceptible to attack by the active, partially acetylated species. This view is supported by the observation of Sri Ram and Maurer[20] that acetylated bovine serum albumin was more rapidly digested by trypsin and chymotrypsin than the unmodified protein, although, in the case of trypsin, a consideration of its known specificity would suggest contrawise. This explanation gains further support from the experiments of Wooton and Hess[25] and Liener[16] which also demonstrated the existence of inactive and active components in autolysates of acetyltrypsin, and which prompted these authors to conclude that it is the hydrolysis of this inactive protein which probably accounts for the liberation of free amino groups without any substantial loss in enzymic activity.

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MARKIERUNG VON INSULIN MIT TRITIUM

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Unter Mitarbeit von
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(Eingegangen den 27. April 1959)

SUMMARY

Labelling of insulin with Tritium

Insulin can be labelled with Tritium by the WILZBACH-method.

Repeated crystallisation or preparative paper electrophoresis followed by crystallisation leads to a preparation of constant specific activity. In paper electrophoresis under various pH conditions as well as during fibril growth followed by reactivation and crystallisation of the reactivated hormone [^{3}H]insulin shows a behaviour equal to that of unlabelled insulin. Columnchromatographic analysis of [^{3}H]insulin hydrolysates reveals a preferential incorporation of the hydrogen isotope into the aromatic amino acids. The bloodsugar-lowering effect of the labelled hormone is fully preserved. Unlabelled insulin causes an isotope dilution effect in the combination of [^{3}H]insulin with rat diaphragm and ox retina *in vitro*.

EINLEITUNG

Während über den Eigenstoffwechsel niedermolekularer Hormone zahlreiche Ergebnisse vorliegen, die mit Hilfe von ^{14}C-, ^{3}H- oder ^{131}J-markierter Substanzen gewonnen wurden, ist die Kenntnis des Schicksals von Proteohormonen im Stoffwechsel beschränkt, da für letztere keine geeigneten Markierungsverfahren bestehen. Biosynthese von Insulin z.B. in Kälberpankreasschnitten *in vitro* aus [^{14}C]Aminosäuren[1,2] sowie bei Ratten *in vivo* aus [^{35}S]Aminosäuren[3] führen lediglich zu geringen spezifischen Aktivitäten des isolierten Hormones. Darüber hinaus können aber molekülfremde Atom-Gruppierungen oder Atome durch chemische Reaktionen in das Insulinmolekül eingeführt werden. So wurden der [^{35}S]Schwefelsäureester des Insulins[4] und das ^{131}J-markierte Derivat[5] dargestellt. Jodiertes Insulin ist in zahlreichen Untersuchungen eingesetzt worden[5]. Die biologische Aktivität der benutzten Präparate, soweit untersucht, bewegt sich, gemessen an der hypoglykämisierenden Wirkung, zwischen 100%[6] und 75%[7]. Die Interpretation der nach Anwendung jodierter Proteine erhaltenen Ergebnisse ist erschwert[8], da eine intravitale Dejodierung ohne Peptidhydrolyse möglich erscheint[9]. Unterschiede im Stoffwechsel jodierter und biosynthetisch markierter Proteine sind beobachtet worden[10].

Wir haben daher untersucht, ob das von WILZBACH[11] entwickelte Verfahren der Inkorporierung von Tritium (^{3}H, β- 0,018 MeV, 12,26 Jahre Halbwertszeit) durch

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* Direktor: Prof. Dr. J. KÜHNAU.

Austausch, speziell für das Proteohormon Insulin anwendbar ist. Über erste Untersuchungsergebnisse wurde von uns bereits kurz berichtet[12, 13]. Von anderer Seite wurde mit Hilfe des WILZBACH-Verfahrens das Protein Lysozym unter Erhaltung der biologischen Aktivität markiert[14].

EXPERIMENTELLES

Markierung mit Tritium

Insulin wurde jeweils in Mengen von 1 g Kristallinsulin* (23 ± 0.5 i.E./mg, 0.62 % Zink) gegenüber einer Gesamtmenge von 2.18 C Tritium bei 0.39 atm. und + 27° über eine Zeit von zwei Wochen exponiert**. Die bei diesen Verfahren beobachteten Anfangsaktivitäten variieren ohne vorangehenden Austausch gegen hydrophile Lösungsmittel zwischen 17.7 und 53 μC/mg Rohprodukt**.

Radioaktivitätsbestimmungen

Die Tritium-Aktivität wurde im fensterlosen Methandurchflusszähler in einer Schichtdicke unterhalb der Selbstabsorption gemessen bzw. die Impulszahlen mit Hilfe von experimentell ermittelten Selbstabsorptionskurven korrigiert. Die Präparate gelangten auf Aluminiumschälchen nach Verdampfung der benutzten Lösungsmittel (0.001 N–0.01 N HCl bzw. CH_3COOH) unter der Infrarotlampe zur Messung. Die Angabe von spezifischen Aktivitäten erfolgt in Impulsen/Min/Gewichtseinheit ± Impulsen/Min für $p = 0.05$. Bei Bezug auf einen Tritiumstandard** beträgt der Wirkungsgrad der Messanordnung *ca.* 30 %. Infolge der ausserordentlich geringen β-Energie des Tritiums und der daraus resultierenden unübersichtlichen Absorptionsverhältnisse im Papier besitzen die Aktivitätsmessungen an Papier lediglich qualitativen Charakter.

Proteinbestimmungen

Die Insulinkonzentration in Lösungen wurde durch Messung der Extinktion bei 278 mμ in 1-cm Schichtdicke unter Bezug auf Kristallinsulinlösungen bekannter Konzentrationen ermittelt. In einem Teil der Untersuchungen wurde die Insulinkonzentration mit Hilfe der von LOWRY[15] angegebenen Methode bestimmt.

Kristallisation von Insulin

Es wurde nach dem Verfahren von ROMAN, SCOTT UND FISHER[15] verfahren. Etwa 0.2 %ige Lösungen von Insulin in 0.1–0.5 N Essigsäure wurden nach Zusatz von 0.07 ml 0.3 %iger Zinkacetatlösung/ml Insulinlösung mit 1 N Ammoniaklösung an der Glaselektrode rasch auf pH 6–7, anschliessend langsam mit 1 N Essigsäure auf pH 5.9 eingestellt, zunächst für 2–3 Stunden bei Zimmertemperatur belassen und dann bei + 4° bis zur vollständigen Kristallisation aufbewahrt. Der durch fraktionierte Zentrifugation bei 100–2000 U./Min gewonnene Kristallniederschlag wurde einmal mit etwa 0.1 M Ammoniumacetatpuffer von pH 5.9 und anschliessend mit destilliertem Wasser gewaschen. Zur Gewinnung von Trockensubstanz erfolgte wiederholte Waschung der Kristalle mit 96 %igem Äthanol und anschliessende Lufttrocknung. Isoelektrische Fällungen wurden im gleichen Ammoniumacetatsystem bei pH 5.2 vorgenommen.

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* Herrn Dr. MITTENZWEI, Hormon-Chemie, München, sei herzlich für die grosszügige Überlassung von Insulin gedankt.

** New England Nuclear Corp., Boston, Mass., USA.

Fibrillenbildung und Reaktivierung

Die Herstellung der fibrillären Modifikation des Insulins durch Animpfen von Insulinlösungen mit Fibrillen sowie dessen Reaktivierung erfolgte nach den Angaben von WAUGH[17,18]. Dabei gelangten Mischungen von elektrophoretisch-gereinigtem $[^{3}\text{H}]$Insulin zu Trägerinsulin wie 1:190 zur Verwendung.

Hydrolyse und Aminosäureanalyse

$[^{3}\text{H}]$Insulin wurde in 5.7 N Salzsäure unter N_2 über 48 Stunden bei 105° hydrolysiert, von überschüssiger Salzsäure im Vakuum über Natriumhydroxyd befreit und nach dem Verfahren von STEIN UND MOORE[19] an Dowex 1- und Dowex 50-Säulen mit ansteigenden Säuregradienten chromatographiert. Konzentrationsbestimmungen erfolgten nach Einengung der Fraktionen zur Trockne (1–10 Torr über Natriumhydroxyd, +50°) nach Wiederaufnahme der Rückstände in 10%igem Pyridin–Wassergemisch mit Hilfe der Ninhydrinreaktion von TROLL UND CANNAN[20]. Aliquote Teile wurden zur Radioaktivitätsbestimmung verwandt. Orientierende Untersuchungen erfolgten papierchromatographisch nach Hydrolyse in folgenden Lösungsmittelsystemen: Pyridin–Amylalkohol–Wasser (7:6:6), *n*-Butanol–Wasser–Eisessig (14:3:3), wassergesättigtes Phenol.

Papierelektrophorese

Analytische Versuche wurden bei einer Feldstärke von 10 V/cm und 2–5 mA/Streifen (Whatman Papier Nr. 1) über 3 Stunden durchgeführt. Folgende Puffersysteme gelangten zur Anwendung: Glykokoll–Salzsäure (pH 2) und Phosphat–Borat (pH 7–9). Die Anfärbung der Proteine auf dem Papier erfolgte nach der von GRASSMANN angegebenen Methode[21]; auf eine quantitative Auswertung wurde aus naheliegenden Gründen verzichtet. In präparativen Ansätzen wurde $[^{3}\text{H}]$Insulin (100–500 mg) in der Anordnung von GRASSMANN[22] (Spinco-Modell CP) bei pH 8.6 im Diäthylbarbitursäure–Natriumdiäthylbarbitursäuresystem (800 V, 58–62 mA, Schleicher u. Schüll-Papier Nr. 470, 4°) aufgearbeitet. $[^{3}\text{H}]$Insulin wurde aus entsprechenden Fraktionen auf zwei Arten isoliert: (a) Isoelektrische Fällung, anschliessende erschöpfende Dialyse bei 4° und Kristallisation. (b) Gefriertrocknung der Fraktionen mit Wiederaufnahme der Trockensubstanz in etwa 0.1 des ursprünglichen Volumens an Wasser, Einstellung von pH 3 und Extraktion der ausgefällten Barbitursäure mit Äther; Fällung des Insulins am isoelektrischen Punkt mit nachfolgender Kristallisation bis zur konstanten spez. Aktivität.

Biologische Teste

Blutzuckerbestimmungen erfolgten nach der Methode von FRANK UND KIRBERGER[23] an männlichen Sprague-Dawley-Ratten (150–200 g Körpergewicht) nach 14 Std. Nahrungskarenz. Insulin wurde in 0.01 N Salzsäure gelöst intravenös appliziert. Diaphragmen 3 Stunden nüchterner Ratten wurden in 2 ml Kaliumphosphat–Natriumchloridpuffer (pH 7, durchgast mit 8% Kohlendioxyd in Sauerstoff) bei 37° und 110 Oszillationen/Min in der Warburg-Apparatur inkubiert. Die Glukosekonzentration des Inkubationsmediums betrug 250 mg%, die Konzentration des Insulins 8.353 µg/Ansatz. Das Verhältnis von markiertem zu unmarkiertem Insulin wurde variiert. Nach 1 Stunde Inkubation wurde in aliquoten Teilen des Mediums die Tritiumaktivität bestimmt. Die Differenz vor und nach Inkubation wurde als Aufnahme/100 mg Diaphragma (Feuchtgewicht) berechnet. In gleicher Weise wie Rattendiaphragmen wurden Rinderretinen inkubiert und die Tritiumaufnahme auf 100 mg Trockengewicht bezogen.

ERGEBNISSE UND DISKUSSION

Die hohe Aktivität des Rohpräparates nach der Exposition gegenüber Tritium lässt das Auftreten zahlreicher Zerstrahlungsprodukte des Insulins erwarten. Daneben dürfte der Hauptanteil des Tritiums in labiler, gegen Wasserstoff austauschbarer Form vorliegen. Hiermit in Übereinstimmung steht das Ergebnis von Dialyseversuchen, s. Fig. 1 und Tabellen I und II. Der rasche Abfall nicht-wasserdampfflüchtiger Radioaktivität entspricht dem Herausdialysieren niedermolekularer Produkte. Da durch Passage des Dialysewassers über einen Kationenaustauscher (Dowex 50 X8 H^+) sich etwa 57 % der nicht-wasserdampfflüchtigen Tritiumaktivität an den Austauscher binden und anschliessend mit NH_4OH wieder eluieren lässt, ist anzunehmen, dass es sich bei diesen Substanzen um Aminosäuren und Peptide handelt, zumal die papierchromatographische Untersuchung des Eluates die Anwesenheit zahlreicher, ninhydrinpositiver, ^3H-markierter Substanzen nachweist. Neben der Beseitigung von

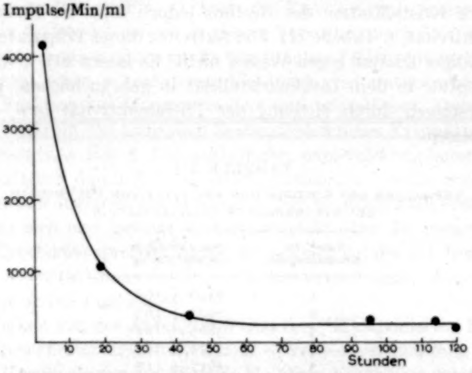

Fig. 1. Nicht wasserdampfflüchtige Bestandteile im Dialysewasser einer [^3H]Insulinlösung im Verlaufe der Dialyse von [^3H]Insulin gegen Wasser bei 4°.

TABELLE I

SPEZIFISCHE AKTIVITÄT VON [^3H] INSULIN NACH BEHANDLUNG MIT WÄSSRIGEN LÖSUNGSMITTELN

Lösungsmittel	*Impulse/Min/µg*
—	11.810·10^3 Impulse/Min*
0.01 N Salzsäure	7.346·10^3 Impulse/Min
0.01 N Salzsäure nach Dialyse gegen Wasser	2.169·10^3 Impulse/Min

* Theoretische Aktivität in der verwendeten Messanordnung unter Zugrundelegung der im Flüssigkeitsscintillationszähler (New England Nucl. Corp.) gemessenen Aktivität ohne vorausgegangenen Austausch gegen hydrophile Lösungsmittel.

TABELLE II

VERTEILUNG DER TRITIUMAKTIVITÄT EINES [^{3}H]INSULINROHPRAEPARATES AUF PROTEIN- UND NICHTPROTEIN ANTEILE

Praeparation	*Total Impulse/Min*
A. Insulin vor Dialyse	$3.149 \cdot 10^6$
B. Insulin nach Dialyse gegen Wasser	$5.783 \cdot 10^5$
C. Nicht-wasserdampf-flüchtige Anteile im Dialysewasser	$1.812 \cdot 10^5$
D. Kationische, nicht-wasserdampf-flüchtige Anteile des Dialysewassers	$1.032 \cdot 10^5$
E. Wasserdampf-flüchtige Anteile des Dialysewassers	$2.389 \cdot 10^6$ (A — Σ BC)

Zerstrahlungsprodukten führt die Dialyse über längere Zeit jedoch zu einem ständigen, langsamen Zerfall des Insulins in der verdünnten Lösung, wie die konstante Aktivität nicht wasserdampfflüchtiger Produkte im Dialysewasser im weiteren Verlauf des Versuches zeigt.

Wiederholte Kristallisation des Insulins jedoch führt zu einem Produkt konstanter spez. Aktivität, s. Tabelle III. Die Aktivität dieses Präparates verändert sich während 5-stündiger Dialyse gegen Wasser nicht. Es lassen sich keine freien Aminosäuren oder Peptide in dem Insulinkristallisat in gebräuchlichen, papierchromatographischen Systemen durch Messung der Tritiumaktivität bzw. der Ninhydrinreaktion nachweisen.

TABELLE III

VERHALTEN DER SPEZIFISCHEN AKTIVITÄT VON [^{3}H]INSULIN BEI WIEDERHOLTER KRISTALLISATION

Anzahl der Kristallisationen	*Spez. Aktivität in Impulse/Min/µg*
1	7346 ± 168
2	2907 ± 105
3	3805 ± 121
4	3423 ± 115
5	3617 ± 117
6	3737 ± 119

Bei Aufbewahrung von [^{3}H]Insulinlösungen konstanter spez. Aktivität in Glasgefässen beobachtet man einen anfänglich raschen, später langsamen Abfall der Aktivität/Volumeneinheit mit Einstellung einer konstanten Endaktivität. Die Feststellung, dass bei Umfüllung der Lösung nach Erreichen der Endaktivität ein erneuter Abfall einsetzt (s. Fig. 2) und die Möglichkeit, durch vorherige Absättigung des Glases mit unmarkiertem Insulin die Aktivitätsminderung pro Volumeneinheit herabzusetzen (Tabelle IV), sprechen für eine Adsorption des Insulins an das Glas.

Papierelektrophoretische Untersuchungen weisen nach, dass [^{3}H]Insulin ausserordentlich intensiv ebenfalls an Papier adsorbiert wird. Unter geeigneten Bedingungen lässt sich auch bei unmarkiertem Insulin eine Adsorption an Papier färberisch nachweisen. Fig. 3 zeigt das Ergebnis der Elektrophorese von [^{3}H]Insulin vor und nach

TABELLE IV

EINFLUSS DER ABSÄTTIGUNG VON GLAS MIT INSULIN AUF DEN AKTIVITÄTSVERLUST EINER [^{3}H]INSULINLÖSUNG

Zeitpunkt in Tagen	*Kontrollgefäss Impulse/Min/ml*	*"Insulin-gesättigtes" Gefäss Impulse/Min*
0	1846	1846
1	885	1311
2	825	1337
3	739	1278

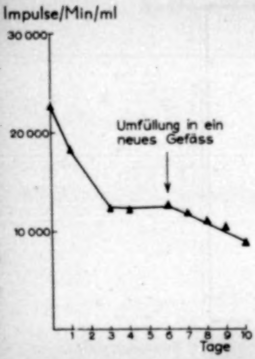

Fig. 2. Aktivitätsverlust einer [^{3}H]Insulinlösung bei Aufbewahrung in einem Glasgefäss.

Kristallisation ohne Zusatz von Trägerinsulin. Die Hauptmasse der Radioaktivität wird an der Auftragungsstelle adsorbiert. Der Zusatz von Trägerinsulin beseitigt diesen Effekt und zeigt das durch die Kristallisation erzielte Ausmass der Beseitigung von Zerstrahlungsprodukten (Fig. 4). Trotz des Zusatzes eines grossen Überschusses von Trägerinsulin findet sich jedoch in diesen Versuchen noch Tritiumaktivität, die nicht der Insulinbande zuzuordnen ist. Es wurde daher [^{3}H]Insulin im kontinuierlichen Durchlaufverfahren einer präparativen Elektrophorese unterworfen und aus der insulinhaltigen Fraktion das Hormon isoliert und kristallisiert. Durch fraktionierte Zentrifugation wurde ein von amorphen Bestandteilen freies Kristallisat der typischen Kristallform erhalten, s. Fig. 5. Die analytische, papierelektrophoretische Kontrolle zeigt ein dem lediglich durch Kristallisation gereinigten Insulin analoges Verhalten des elektrophoretisch abgetrennten Hormones (Fig. 6). Auch nach elektrophoretischer Reinigung findet sich eine geringe Tritiumaktivität über die gesamte Laufstrecke verteilt. Diese Ergebnisse sprechen dafür, dass es sich bei der der Insulinbande nicht zuzuordnenden Aktivität um an das Papier adsorbiertes Insulin handelt und nicht um eine Verunreinigung des Präparates.

In Abhängigkeit von der Anfangsaktivität des [^{3}H]exponierten Insulins und der Dauer der Aufbewahrung des Rohpräparates werden verschiedene Aktivitäten des gereinigten [^{3}H]Insulins gefunden (Tabelle V). Diese Ergebnisse machen eine interne

TABELLE V

SPEZIFISCHE AKTIVITÄTEN VERSCHIEDENER [^{3}H] INSULIN-PRÄPARATE

Aktivitäten in Impulse/Min/mg	*Präparation*	*Zeitpunkt der Untersuchung*
$7.35 \cdot 10^6$	Rohprodukt (I)	4 Monate nach Exposition
$3.40 \cdot 10^6$	Kristallisation bis zur konst. spez. Aktivität (I)	4 Monate nach Exposition
$1.59 \cdot 10^6$	Kristallisation bis zur konst. spez. Aktivität (I)	18 Monate nach Exposition
$0.45 \cdot 10^6$	Papierelektrophoretische Reinigung (I)	18 Monate nach Exposition
$9.96 \cdot 10^6$	Rohprodukt (II)	6 Monate nach Exposition
$2.49 \cdot 10^6$	Kristallisation bis zur konst. spez. Aktivität (II)	6 Monate nach Exposition
$0.79 \cdot 10^6$	Papierelektrophoretische Reinigung (II)	6 Monate nach Exposition

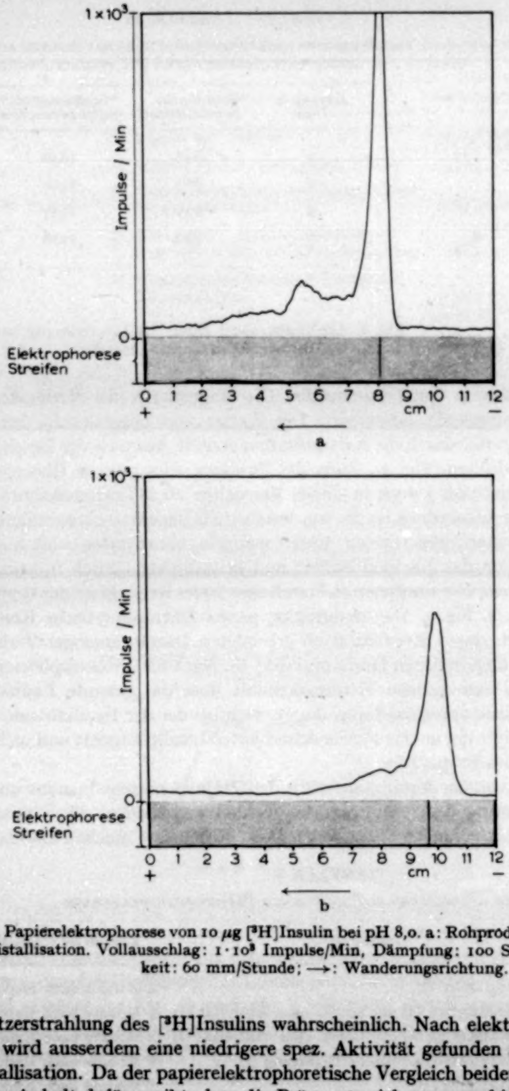

Fig. 3. Papierelektrophorese von 10 μg [^{3}H]Insulin bei pH 8,0. a: Rohprodukt; b: nach dreimaliger Umkristallisation. Vollausschlag: 1 · 10^3 Impulse/Min, Dämpfung: 100 Sek; Schreibgeschwindigkeit: 60 mm/Stunde; →: Wanderungsrichtung.

Selbstzerstrahlung des [^{3}H]Insulins wahrscheinlich. Nach elektrophoretischer Reinigung wird ausserdem eine niedrigere spez. Aktivität gefunden als nach wiederholter Kristallisation. Da der papierelektrophoretische Vergleich beider Kristallisate jedoch keinen Anhalt dafür ergibt, dass die Präparate sich aus verschiedenen Komponenten zusammensetzen, muss angenommen werden, dass der lange Verbleib des [^{3}H]Insulins in einer Lösung von pH 8.6 unter Bedingungen, die eine Oxydation des Insulins

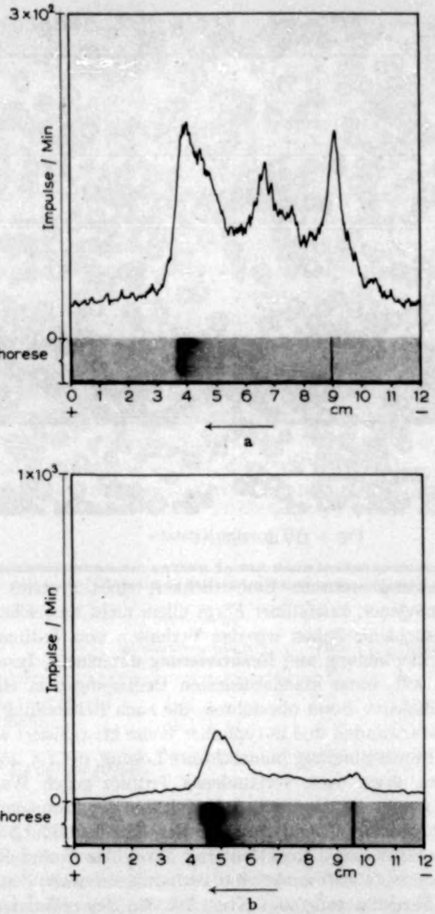

Fig. 4. Papierelektrophorese einer Mischung von 10 µg [^{3}H]Insulin und 900 µg unmarkiertem Insulin bei pH 8,0. a: Rohprodukt; b: nach dreimaliger Umkristallisation. Vollausschlag: a = 3·10/ Impulse/Min; b = $1\cdot10^3$ Impulse/Min; Dämpfung: 100 Sek; Schreibgeschwindigkeit: 60 mm/Stunde; →: Wanderungsrichtung.

begünstigen, zu einer Denaturierung von [^{3}H]Insulin-Molekülspezies höherer spez. Aktivität geführt hat oder unter diesen Bedingungen ein zusätzlicher Rückaustausch von Tritium gegen Wasserstoff stattgefunden hat.

Unter Zugrundelegung der gemessenen spez. Aktivitäten des gereinigten [^{3}H]-Insulins ist der Wasserstoff des Insulins in der Gröszenordnung von 10^{-5} – 10^{-4} % durch Tritium ersetzt worden.

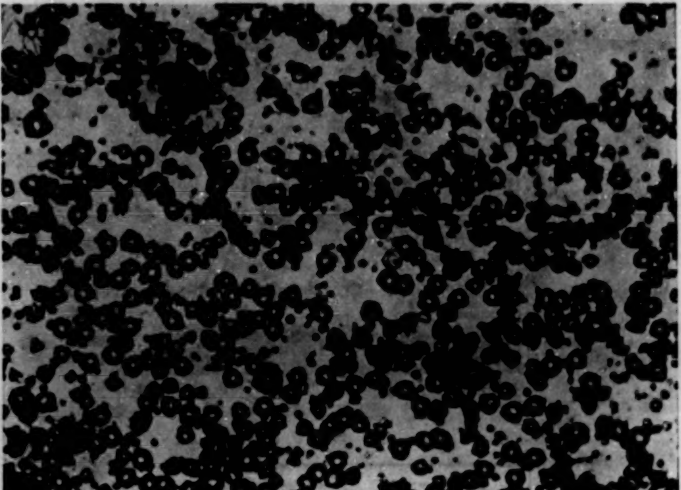

Fig. 5. [^{3}H]Insulin Kristalle.

Da die papierelektrophoretische Einheitlichkeit eines Proteins sowie dessen Darstellbarkeit in homogener, kristalliner Form allein nicht ausreichend ist für die Annahme der Einheitlichkeit, haben wir das Verhalten von Tritiuminsulin unter Bedingungen der Fibrillenbildung und Reaktivierung untersucht. Insulin lässt sich, wie WAUGH[17] gezeigt hat, unter standardisierten Bedingungen in eine unlösliche, fibrilläre, biologische inaktive Form überführen, die nach Behandlung mit Alkali in natives Insulin zurückverwandelt und in typischer Weise kristallisiert werden kann[18]. Die Bedingungen der Fibrillenbildung (mineralsaure Lösung, pH 1.5, 40°) sind gleichzeitig geeignet, labiles, etwa noch vorhandenes Tritium gegen Wasserstoff auszutauschen. Tabelle VI zeigt die spezifischen Aktivitäten der Lösungen von kristallisierten [^{3}H]Insulin-Trägerinsulin-Gemischen vor der Fibrillenbildung und nach Fibrillenbildung mit anschliessender Reaktivierung durch Alkali und Kristallisation. 92.4 ± 4.4 % der eingesetzten Tritiumaktivität verhalten sich unter den Bedingungen der Fibrillenbildung, Reaktivierung und Kristallisation der reaktivierten Fibrillen

TABELLE VI

VERHALTEN DER SPEZIFISCHEN AKTIVITÄT VON GEMISCHEN AUS ELEKTROPHORETISCH GEREINIGTEM [^{3}H]INSULIN UND INAKTIVEM INSULIN UNTER BEDINGUNGEN DER FIBRILLENBILDUNG

Art der Insulinpraeparation	*Spez. Aktivität in Impulse/Min/mg*
Kristallisat vor Fibrillenbildung	4170 ± 185
Kristallisat nach Fibrillenbildung und Reaktivierung	3850 ± 186

wie natives Insulin. Diese Ergebnisse machen eine weitgehende Identität von Tritiuminsulin und unmarkiertem Insulin wahrscheinlich.

Bei der Untersuchung der Verteilung des Einbaues von Tritium in die verschiedenen Aminosäuren des Insulins wurde als analytische Methode das von STEIN UND MOORE[19] angegebene Verfahren der Chromatographie an Dowex 50 X 8 H^+ mit steigendem Salzsäuregradienten gewählt. Für die Beantwortung unserer Fragestellung erscheint dieses Vorgehen im Vergleich zu den mit Puffergemischen arbeitenden Verfahren trotz der bei letzteren besseren Trennung geeigneter, da eine Messung der Radioaktivität der Aminosäuren im Hinblick auf die geringe β-Energie des Tritiums aus methodischen Gründen möglichst in unendlich dünner Schicht erfolgen muss, dies jedoch bei der verwendeten Probenbereitung und Messung in Gegenwart von Puffersalzen nicht realisierbar ist. Fig. 7 zeigt, dass ein Einbau von Tritium in verschiedene Aminosäuren stattfindet, wobei die aromatischen Aminosäuren Phenylalanin, Tyrosin und Histidin bevorzugt sind, während die Monoaminodicarbonsäuren und Hydroxyaminosäuren nur geringere Aktivitäten aufweisen. Auffällig erscheint die in mehrereren Chromatogrammen beobachtete Aufspaltung von Radioaktivitätsgipfeln bzw. deren Verschiebung gegenüber den Ninhydrinmaxima. Es handelt sich dabei möglicherweise um Isotopeneffekte unterschiedlich markierter Aminosäuren der gleichen Spezies.

Die Markierung des Insulins führt, gemessen an der Blutzuckeraktivität des gereinigten [^{3}H]Insulins, zu keinem Verlust der biologischen Aktivität (Tabelle VII).

TABELLE VII

BLUTZUCKERSENKUNG BEI RATTEN 15 MIN NACH INTRAVENÖSER INJEKTION VON [^{3}H]INSULIN BZW. UNMARKIERTEM INSULIN*

	Mittelwert des Blutzuckerabfalles und Standardabweichung in % des Ausgangsblutzuckers	*Anzahl der Tiere*	*Signifikanz der Mittelwertsdifferenzen*
Insulin (25 I.E./mg)	33.0 ± 9	8	Keine Differenz
[^{3}H]Insulin	33.5 ± 13	12	

* 0.4 µg/100 g Ratte.

Allerdings würde das Vorhandensein von geringsten, biologisch inaktiven Anteilen hoher spezifischer Radioaktivität in einem derartigen Test nicht aufgedeckt werden. Es wurde daher ein weiteres Merkmal zur biologischen Charakterisierung des [^{3}H]Insulins herangezogen. Insulin wird, wie STADIE[4] gezeigt hat, in spezifischer Weise von verschiedenen Geweben gebunden. Es wurde daher untersucht, ob die Tritiumaktivität [^{3}H]Insulins von Rattendiaphragmen oder Rinderretinen in gleicher Weise gebunden wird wie unmarkiertes Insulin. Wenn die Bindung der Tritiumaktivität an die spezifischen Gewebsstrukturen in gleicher Weise erfolgen würde wie die des Insulins, müsste bei partiellem Ersatz von [^{3}H]Insulin durch unmarkiertes Insulin bei konstanter Konzentration von Gesamtinsulin im Ansatz ein echter Isotopenverdünnungseffekt bezüglich der Bindung an die Gewebe nachweisbar sein. Die Figs. 8 und 9 zeigen, dass die sich aus dem Verdünnungsgrad des [^{3}H]Insulins durch unmarkiertes Insulin errechnende Aufnahme von Tritiumaktivität den experimentell bestimmten Werten entspricht.

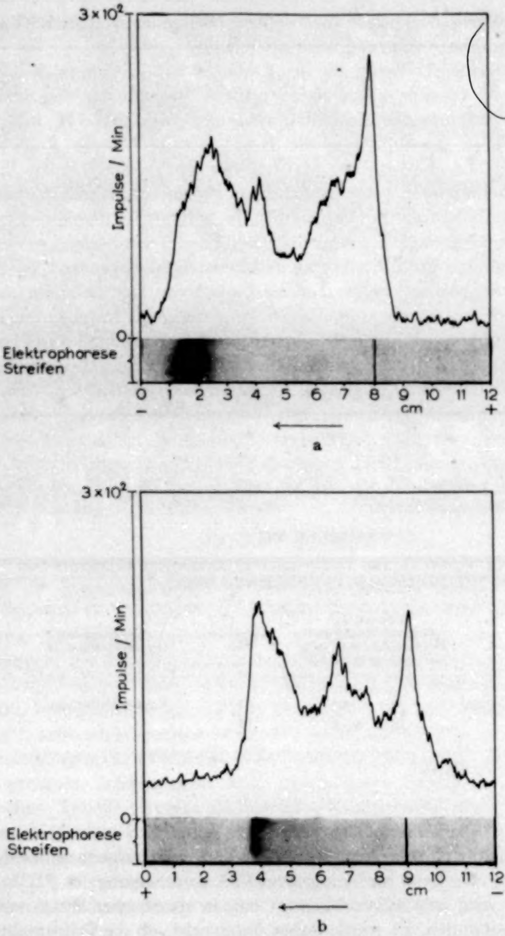

Fig. 6; Legende s. S. 99.

Damit ist bewiesen, dass unter den angewandten Versuchsbedingungen die Bindung von Tritiumaktivität des tritiierten Insulins an die Gewebe den gleichen Gesetzmässigkeiten unterliegt wie unmarkiertes Insulin. Da [^{3}H]Insulin sich bei der Kristallisation, Papierelektrophorese und Fibrillenbildung sowie in seiner hypoglykämischen Wirkung als auch in der Bindung an Gewebe wie unmarkiertes Insulin verhält, erscheint seine Verwendung als tracer in biologischen Versuchen berechtigt.

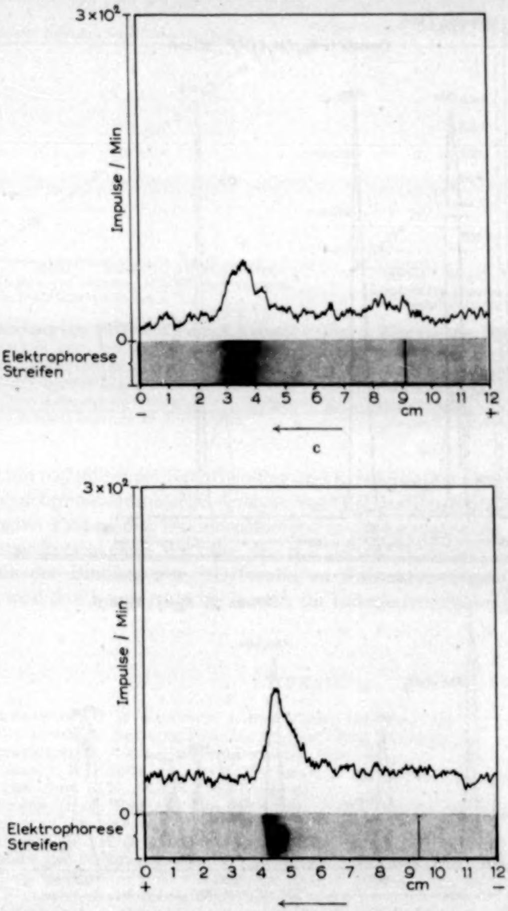

Fig. 6. Papierelektrophorese von Mischungen aus 10 μg [³H]Insulin und 900 μg unmarkiertem Insulin. a: Rohprodukt pH 2,0; b: Rohprodukt pH 8,0; c: nach elektrophoretischer Reinigung, pH 2.0; d: nach elektrophoretischer Reinigung pH 8.0. Vollausschlag: $3 \cdot 10^2$ Impulse/Min, Dämpfung: 100 Sek; Schreibgeschwindigkeit: 60 mm/Stunde; ⟶: Wanderungsrichtung.

DANK

Die Untersuchungen wurden vom Bundesministerium für Atomkernenergie und Wasserwirtschaft gefördert.

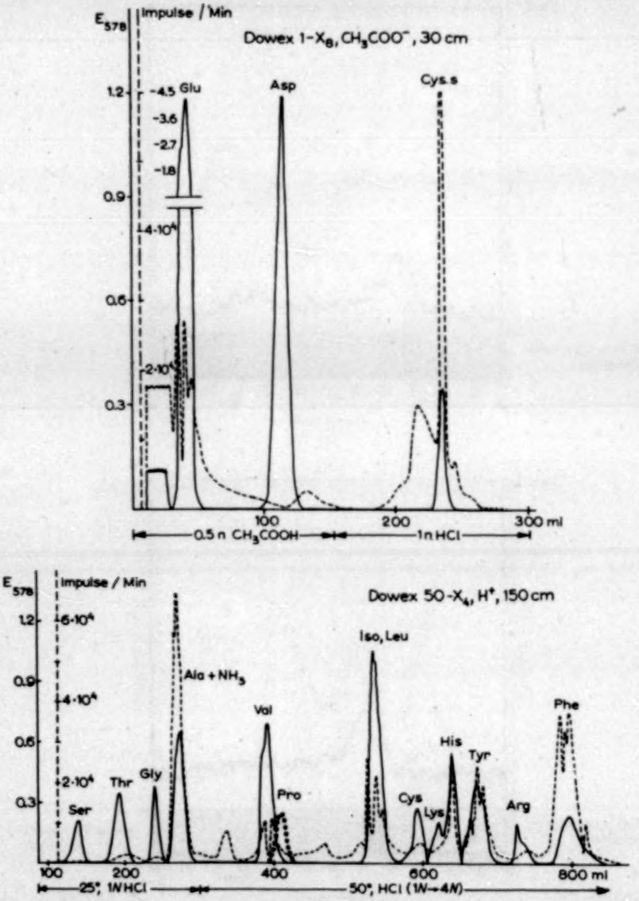

Fig. 7. Chromatographie eines Hydrolysates von mehrfach umkristallisiertem [^{3}H]Insulin an Ionenaustauschersäulen nach MOORE UND STEIN[19]. ——— = $E_{578\,m\mu}$; – – – – = Impulse/Min/Volumeneinheit; ········ = $E_{436\,m\mu}$.

ZUSAMMENFASSUNG

Insulin lässt sich im WILZBACH-Verfahren mit Tritium markieren.

Durch wiederholte Kristallisation oder durch präparative Papierelektrophorese mit nachfolgender Kristallisation kann das Hormon als Präparat mit konstanter spezifischer Aktivität erhalten werden. Tritiuminsulin verhält sich in der Papierelektrophorese unter verschiedenen pH-Bedingungen sowie im Verlaufe der Fibrillen-

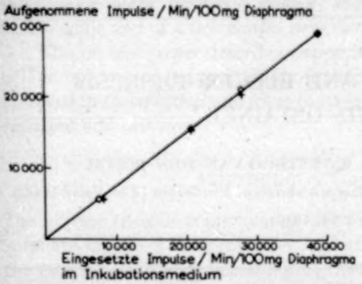

Fig. 8. Bindung von [^{3}H]Insulin durch Rattendiaphragma *in vitro* bei Variation des Verhältnisses von markiertem zu unmarkiertem Insulin und gleichbleibender Gesamtkonzentration von Insulin. ◇ = Berechnete Aufnahme; + = Experimentell bestimmte Aufnahme.

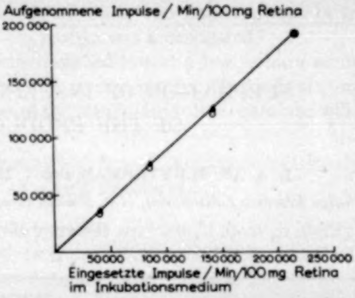

Fig. 9. Bindung von [^{3}H]Insulin durch Rinderretina *in vitro* bei Variation des Verhältnisses von markiertem zu unmarkiertem Insulin und gleichbleibender Gesamtinsulinkonzentration. ▲ = Berechnete Aufnahme; ○ = Experimentell bestimmte Aufnahme.

bildung mit nachfolgender Reaktivierung und Kristallisation wie unmarkiertes Insulin. Die säulenchromatographische Analyse von [^{3}H]Insulin-Hydrolysaten weist einen bevorzugten Einbau des Wasserstoffisotops in die aromatischen Aminosäuren nach. Die blutzuckersenkende Wirkung des markierten Hormones bleibt voll erhalten. Bezüglich der Bindung von [^{3}H]Insulin an Rattendiaphragma oder Rinderretina *in vitro* wird durch unmarkiertes Insulin ein Isotopenverdünnungseffekt bewirkt.

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SULPHATION OF INSULIN AND ELECTROPHORESIS OF THE PRODUCTS OBTAINED[*]

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SUMMARY

The SO_3 complexes of a few tertiary amines were tested for their ability to introduce SO_3 groups into insulin. Pyridinium sulphonic acid was found to be the most suitable one. This reagent is not hydrolyzed immediately and therefore is able to exert a sulphating action for some time after being dissolved in the reaction medium.

"Sulphate" groups[**] are introduced into amino groups and, at a slower rate into tyrosine and histidine residues. By variation of the reaction conditions, insulin preparations of various sulphate content were prepared and subjected to paper electrophoresis at pH 1.7 (Fig. 3). A total number of 13 well defined, approximately equidistant bands could be observed, corresponding to insulin molecules carrying different electrical charges and covering a range from + 6 units (native insulin) to — 6 units (completely sulphated insulin). The intensities of the bands in the electropherogram of a partly sulphated insulin could be related to the different reactivities of the groups concerned.

The biological activity of the preparations decreased with increasing sulphate content.

INTRODUCTION

The attachment of sulphate or sulphonate groups to protein molecules can be an effective method of obtaining protein derivatives of low isoelectric point. This has been accomplished by treating proteins with concentrated sulphuric acid at low temperatures[1,2], or with the reaction product of chlorosulphonic acid and pyridine under anhydrous conditions at 70–80° [3]. The former reaction converts aliphatic and aromatic hydroxyl groups to acid sulphate esters. The latter reaction involves the same groups and, in addition, the amino groups and sulphhydryl groups.

Far milder conditions for sulphation than those mentioned above have been described by BAUMGARTEN[4]. Pyridinium sulphonic acid (the addition complex of pyridine and sulphur trioxide) in aqueous potassium carbonate at 10° was found to sulphate aromatic hydroxyl groups, imidazole moieties, and amino groups of free amino acids and of a few simple peptides. No protein was investigated.

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[**] Although both sulphate monoester groups and N-sulphonic acid groups occur in the products, for the sake of brevity the terms "sulphation" and "sulphate groups" are generally used in this paper. This choice was made because both types of groups are converted into inorganic sulphate by acid hydrolysis.

The present paper describes experiments designed to test a few tertiary amine–SO_3 complexes for their suitability to sulphate the protein insulin. The kinds of groups involved in the reaction and some properties of the insulin derivatives obtained will be reported and discussed.

SULPHATING ACTION OF A FEW REAGENTS

The sulphur trioxide complexes of four tertiary amines were tested for their ability to sulphate insulin. The preparative and analytical procedures were those described in the experimental part. A few representative data are shown in Table I.

TABLE I

REACTIVITY OF AMINE – SO_3 COMPLEXES AT pH 10, 0°

Amine	*Amount of reagent g/g insulin*	*Sulphate groups introduced per insulin molecule*
Pyridine	0.2	4.2
Pyridine	1.0	6.8
Dimethylaniline	0.3	3.3
Dimethylaniline	1.0	4.9
Dimethylaniline	2.5	5.6
Triethylamine	1.0*	≤ 0.2
Trimethylamine	1.0*	0.7
Trimethylamine	1.0**	3.1

* Reaction period 3 h.
** Reaction period 24 h.

It may be concluded from Table I that, of the reagents tested, pyridinium sulphonic acid (PS)* is the most suitable one. The investigations we report on here were therefore carried out with this reagent.

The sulphonates of trimethylamine and triethylamine apparently react at a slow rate. This may be due to the stability of these complexes under the present conditions, judging from the very slow drop in pH observed after the addition of the reagents to the aqueous reaction mixture. This drop is caused mainly by the hydrolysis of the compounds. Even at the end of 24 hours only a small part of the reagent appeared to be hydrolyzed.

The hydrolysis of dimethylanilinium sulphonic acid, on the other hand, occurs in a few minutes; PS occupies an intermediate position. The different effectiveness of these two compounds as regards the sulphation reactions cannot be explained solely by the different stability, since it is the ratio of hydrolysis rate and sulphation rate that is the deciding factor. It may also be due to a difference in solubility of the suspended reagents. Naturally, the solubility of the complexes themselves cannot be measured very well, but those of the parent amines differ a great deal, since dimethylaniline is only slightly soluble in water while pyridine can be mixed in all proportions. This difference may be reflected in the solubilities of the complexes. The following experiment indicates that it may be the dissolved part of the reagent that exerts the sulphating action.

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* Abbreviations: PS, pyridinium sulphonic acid; DNFB, 2,4-dinitro fluoro benzene; DNP, 2,4-dinitrophenyl moiety.

In 10 ml borate buffer pH 10.0, 200 mg PS was suspended at room temperature. The reaction mixture was stirred and the pH kept at the same level for one hour. The small amount of reagent remaining was then filtered off. The filtrate was cooled to 0° and 200 mg insulin added. The reaction mixture was stirred and kept at pH 10.0 for one hour. The product was separated in the usual manner and found to contain an average number of two SO_3 groups per molecule.

Apparently PS is not hydrolysed immediately on being dissolved but is still reactive for some time.

THE GROUPS INVOLVED IN THE SULPHATION REACTION

Five samples of insulin were subjected to the action of different amounts of PS and the sulphate content of the products determined by the methods described in the experimental part of this paper. The more reagent was applied, the more sulphate groups were found to be introduced. The most sulphated product contained about 8 ½ sulphate groups per molecule (Table III, Fig. 1).

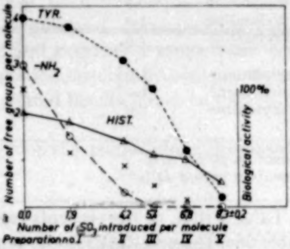

Fig. 1. Distribution of sulphate groups over tyrosine, histidine and amino groups in five sulphated insulin preparations.

(*a*) *Qualitative investigation*

In order to establish in a qualitative manner which groups are involved in the sulphation reaction, a preparation of high sulphate content (prep. V, Fig. 1) was subjected to the action of DNFB, the resulting product hydrolysed and the hydrolysate applied to a paper chromatogram. On the same strip, solutions of tyrosine, histidine, hydrolysates of native insulin, sulphated insulin (preparation V) and the DNP derivative of native insulin were applied. The strip was developed and stained as described in the experimental part. The result is shown in Fig. 2. The hydrolysate of insulin contains histidine and so does the hydrolysate of sulphated insulin (preparation V), because the N-sulphonate group, if present, is removed by hydrolysis. Virtually no histidine is found in dinitrophenyl insulin, because the imidazole nucleus is modified by the alkylation reaction. On the other hand, sulphated insulin, after reaction with dinitrofluoro benzene and hydrolysis, does contain histidine, apparently because the imidazole group was shielded from the interaction with dinitrofluoro benzene by a sulphonate group. Hence *histidine* participates in the sulphation reaction.

In the same manner the participation of *tyrosine* is apparent. Finally, Fig. 2 demonstrates the complete sulphation of the *ε-amino group of lysine*. The hydrolysate of DNP insulin produced the yellow spot of ε-DNP lysine (Fig. 2, L); the hydrolysate of DNP insulin sulphate, however, did not contain any of this compound, apparently because the ε-NH_2 group was shielded by a SO_3 group against the interaction of DNFB. Even when 5 times as much hydrolysate was applied to the chromatogram no ε-DNP lysine could be detected*. This indicates that amino groups are completely sulphated by PS. Hence, it may be concluded that *tyrosine*, *histidine* and *amino groups* participate in the sulphation reaction.

Fig. 2. Lower part of paper chromatogram of I tyrosine, II histidine, III hydrolysate of insulin, IV hydrolysate of sulphated insulin, V hydrolysate of DNP insulin, VI hydrolysate of DNP sulphated insulin.

(*b*) *Quantitative investigation*

The distribution of the sulphate groups over the three types of groups mentioned above was established by quantitative determination of free amino groups and free phenolic groups, the former groups by amino nitrogen determination according to Van Slyke, the latter groups by u.v. absorption in a strongly alkaline medium, as described in EXPERIMENTAL. The sulphate groups in excess of amino groups and phenolic groups were considered to be attached to histidine. The results are given in Fig. 1. The data concerning histidine are, of course, of an approximate nature only, but serve to show the trend.

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* Small amounts of yellow compounds of unknown identity were present. These could be plainly distinguished from ε-DNP lysine.

The degree of sulphation of histidine in preparation V was also derived in a more direct manner from histidine determinations in the hydrolysates of Fig. 2, (for details see EXPERIMENTAL). It appeared that 0.6 out of the 2.0 histidine residues of preparation V had been free and reacted with DNFB. This compares favourably with the 0.5 free histidine residues recorded in Fig. 1. Hence all sulphate groups of preparation V are accounted for in terms of tyrosine, histidine and amino groups and no other groups of insulin are involved in the sulphation reaction.

It may be concluded that sulphation of insulin by the method described in this paper is not a specific reaction, since it acts upon three kinds of groups*. In insulin the same groups react as those of the free amino acids as found by BAUMGARTEN.

Before going on to the next paragraph one should note that the amino groups react at a higher rate than the other groups do, as shown in Fig. 1.

ELECTROPHORETIC PROPERTIES OF SULPHATED INSULIN PREPARATIONS

In acid medium, insulin is known to carry a net charge of + 6 units. It will be clear that the introduction of sulphate groups will affect this charge, making the molecules less positive or even negative, depending upon the number and site of the sulphate groups introduced.

The result of a sulphation experiment, in which, for example, on the average 2 sulphate groups are introduced per insulin molecule, will be a mixture of various molecular species, *viz.* insulin molecules carrying zero, one, two and more sulphate groups. It is therefore a mixture of molecules of almost the same molecular weight differing in electrical charge by steps of one electronic charge. These species, present in such a mixture, will manifest themselves in electrophoretic experiments on paper, giving rise to bands which, in a first approximation, will be equidistant. This has actually been found and it will be shown that the results of electrophoretic experiments are in perfect agreement with the analytical results described in the preceding paragraph.

The charge of + 6 units of insulin in acid medium is known to be due to 3 amino groups, 2 histidine residues and one arginine residue[5]. The introduction of SO_3 groups into the protein molecule causes a shift in the charge of the groups that participate in the reaction. These groups and their charge are tabulated in Table II, prior to and after sulphation.

Monophenol sulphate and aminosulphonic acid are known to be strong acids. Therefore the corresponding groups in protein derivatives will carry a negative charge, as indicated in Table II.

Sulphonation of the imidazole nucleus was assumed to produce a compound with no net charge, since the nitrogen atom not involved in the reaction might retain its basic properties. This was verified in the following manner. Benzimidazole and benzimidazole N-sulphonic acid were subjected to paper electrophoresis in 33 % acetic acid. Glucose was run at the same time for the purpose of marking the point of zero charge. It was found that benzimidazole had travelled a certain distance from the glucose spot because of its positive charge; the sulphonic acid, on the other hand,

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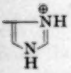
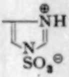
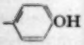
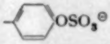
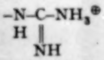
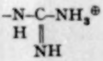
* Possibly upon four kinds of groups in SH-containing proteins, as was derived from sulphation experiments on glutathione in argon atmosphere, which indicated sulphation of the sulphhydryl group (see EXPERIMENTAL).

TABLE II

CHARGE OF INSULIN GROUPS AT pH 1,7

Native insulin				*Completely sulfated insulin*				
	n	*e*	*ne*		*n*	*e*	*ne*	*Δe*
$-NH_3^{\oplus}$	3	+ 1	+ 3	$-NH-SO_3^{\ominus}$	3	— 1	— 3	— 2
	2	+ 1	+ 2		2	0	0	— 1
	4	0	0		4	— 1	— 4	— 1
 $-\underset{\mathrm{H}}{\mathrm{N}}-\underset{\underset{\mathrm{NH}}{\|}}{\mathrm{C}}-\mathrm{NH}_3^{\oplus}$	1	+ 1	+ 1	 $-\underset{\mathrm{H}}{\mathrm{N}}-\underset{\underset{\mathrm{NH}}{\|}}{\mathrm{C}}-\mathrm{NH}_3^{\oplus}$	1	+ 1	+ 1	0
		Total charge	+ 6			Total charge	— 6	

n number of groups per insulin molecule, *e* charge of each group, *Δe* shift in charge on sulphation of each group.

was scarcely displaced and therefore carried no overall charge. Hence, the assumption concerning the converted imidazole nucleus, as indicated in Table II, corresponds with actual fact.

These data may be applied to the preparations of Fig. 1. Preparation I probably consists of a mixture of some native insulin molecules and molecules having one, two or all three of its amino groups converted to sulphamate groups. Taking into account the shift of — 2 charges for each sulphamate group, these four kinds of molecules will carry + 6, + 4, + 2 and 0 charges. A few of each kind will carry a SO_3 group also on a tyrosine or histidine residue and therefore carry + 5, + 3, + 1 and — 1 charges, since for sulphation of a tyrosine or histidine residue a further shift of — 1 unit occurs. Hence one may expect that an electropherogram of preparation I will show four relatively strong bands due to molecules carrying an even number of charges and four relatively weak bands due to molecules carrying an uneven number of charges. This has actually been found (Fig. 3, A). Clearly the even-numbered bands are relatively stronger than the uneven-numbered ones. The mobilities of the first four bands are similar to those that have previously been observed with partially acetylated insulin[6].

In the other preparations recorded in Fig. 1 most of the amino groups have reacted. The different charge species differ mainly in the degree of reaction of their tyrosine and histidine residues. Each reaction with one of these groups results in a shift of only one charge. Therefore no alternation of weak and strong bands can be expected, as was in fact observed (Fig. 3, B–E).

As indicated in Table II a completely sulphated insulin molecule may carry a net charge of — 6 units. Band "— 6" of Fig. 3 E corresponds to this compound. Hence all of the 13 bands corresponding to the charge species + 6 to — 6 have now been found.

It is apparent from the data of Fig. 1 that preparation V also contains some

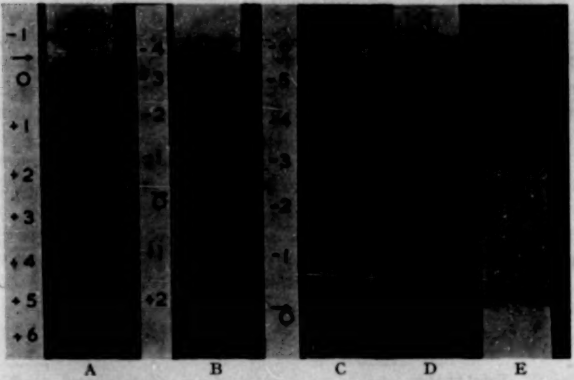


Fig. 3. Electropherograms of five insulin preparations of increasing sulphate content. A and B, 16 h, 4.4 V/cm. C, D and E, 16 h, 6.6 V/cm. Buffers: 33 % acetic acid pH 1.7. → point of application. A 50 μl 3 % protein solution, B–E 50 μl 1 % protein solution applied to the starting point.

insulin molecules having one or two free tyrosine and/or histidine residues. The bands "— 5" and "— 4" of Fig. 3 E correspond to these kinds of molecules*.

It may be concluded that the analytical data of Fig. 1 correspond very well with the electropherograms of Fig. 3.

The bands obtained are as sharp as those of native insulin at comparable concentrations. This may indicate that the electrophoretic mobility of a protein molecule is not greatly affected by whether a charged group is situated at one site or another on the molecule.

Clearly, sulphation is an effective method of obtaining protein derivatives of low isoelectric point. Even after limited sulphation of insulin (preparation II) the mean isoelectric point of the product is below pH 1.7, as shown by Fig. 3 B, since band "— 1", which is the strongest one, travels to the anode at pH 1.7.

Finally, a few remarks concerning the electrophoretic mobility of the compounds. One might expect that, in paper electrophoresis, completely sulphated insulin (charge of — 6 units) would travel to the anode at the same rate as native insulin (charge of +6 units) travels to the kathode. In fact, however, sulphated insulin moves at a lower rate than native insulin does, as can be observed by comparing strips A and E of Fig. 4. The bands "+ 6" of Fig. 3 A and "— 6" of Fig. 3 B travelled at approximately the same distance, but the former strip was run at 4.4 V/cm, the latter at 6.6 V/cm. Among other factors this lower mobility may be due to incomplete dissociation of the sulphate groups at pH 1.7.

Incomplete dissociation may also explain the electrophoretic behaviour of band "0". If glucose is run at the same time in order to mark the point of zero mobility, the glucose band does not coincide with band "0" but is found near the middle, between the bands "0" and "— 1". Thus band "0" actually carries a charge of about — ½ unit. The three sulphamate groups therefore seem to decrease the charge not by 6 units, as assumed in Table II, but by about 5½ units. If the groups were not dissociated at all, the decrease would have been 3 units. Consequently, the actual decrease of 5 ½ units could be explained by a dissociation of 80 % of the sulphamate groups at pH 1.7. This corresponds reasonably well with 80 % dissociation of sulphamic acid at pH 1.6, as calculated from dissociation data of solutions of the latter acid[7]. However, proton binding on other sites may explain the phenomena just as well.

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* A faint ill defined band of higher mobility than band "-6" is probably due to impurities.

BIOLOGICAL ACTIVITY

From the available data, some conclusions concerning the biological activity of insulin may be made. By increasing the degree of sulphation the biological activity of insulin decreases (Fig. 1). When most of the amino groups are converted (preparation II) a certain amount of activity still remains. This indicates that the amino groups are not essential for activity. The same conclusion has been drawn by FRAENKEL-CONRAT[8]. He found 80 % activity in an insulin preparation whose amino groups had been almost completely acetylated by means of acetic anhydride. This fact has been confirmed by us[9].

The data of preparations III and IV indicate that at least one tyrosine or histidine is not essential for activity. Preparation III has about 1/6 of the activity of native insulin (or 1/5 of the activity of acetyl insulin). The only fraction which may contain no sulphate groups on tyrosine or histidine, band "0" of Fig. 3 C, is certainly smaller than 1/6 of all fractions as judged from the electropherogram. Therefore at least one of the other fractions must have some activity.

The same line of reasoning may be followed for preparation IV, which has 1/16 of the activity of native insulin. Fig. 3 D shows that not a trace of band "0" was present. Even band "— 1" may be present in smaller amounts than 1/16 of the other fractions, indicating that even introduction of two sulphate groups on tyrosine and/or histidine residues may not lead to total loss of activity.

Non-essentiality of a part of the tyrosines is known already from iodination experiments[3] and sulphation with sulphuric acid in the cold[2]. Similar knowledge about histidine must await other experiments.

Finally, one should not overlook the possibility that an increase in negative charge, as demonstrated in the present preparations, may in itself, cause a decrease of activity.

EXPERIMENTAL

The crystalline insulin, supplied by Philips-Roxane, Netherlands, batch 04/05 A5-V, biological activity 24.5 I.U./mg, contained 8 % moisture. After drying, the analytical data were those presented in Table I.

Preparation of sulphur trioxide complexes. Pyridinium sulphonic acid was prepared from pyridine and chlorosulphonic acid[10]. The other complexes were obtained by adding a solution of sulphur trioxide in chloroform to a solution of the amine in chloroform cooled by ice–salt mixture[11].

Sulphation conditions. 200 mg crystalline insulin is dissolved in 10 ml 0.1 *M* borax at 0° and the solution adjusted to pH 10.0 with 1.5 *N* KOH from a syringe whilst vigorously stirring with a magnetic stirrer. In small portions 50–200 mg PS is suspended in the reaction mixture at intervals of about 15 min for a period of from one to three hours. The pH is maintained between 9.9 and 10.1 by slow addition of

KOH. The reaction is considered to be completed when all reagent is dissolved and the pH remains constant. The total reaction time was about 8 h for the largest amount of reagent applied. The reaction mixture is acidified to pH 5.5, and dialysed at room temperature against several changes of distilled water under stirring conditions for 65 h, and lyophylised. Yields were almost quantitative. A second 65 h period of dialysis did not decrease the total sulphur content of a few preparations.

Sulphation at pH 11.0 was carried out in 0.1 *M* soda buffer, the other conditions remaining the same.

Glutathione, 20 mg, was sulphated in 10 ml borax buffer pH 10.0 by 200 mg PS. In order to prevent oxidation a current of argon was passed through during the reaction. After the reaction was completed the mixture was acidified to pH 7 and diluted to 50 ml. Samples of 1 ml were used for amperometric titration in "tris" buffer using $AgNO_3$. No sulphhydryl groups remained.

A "blank sulphation" was carried out by adding pyridine plus sulphuric acid, instead of PS, the other reaction conditions remaining the same. In this way the sulphhydryl content was decreased by 15 %.

Dinitrophenylation. DNP insulin was prepared from zinc-free insulin according to Sanger[8]. Samples of sulphated insulin were treated in the following manner:

50 mg of preparation V (Fig. 1) and 100 mg sodium bicarbonate were dissolved in 1 ml water. 100 mg DNFB, dissolved in 2 ml alcohol, was added and the mixture shaken for 2 h at room temperature. Since no insoluble product separated after acidification with 2 drops of concentrated HCl, the reaction mixture was diluted with 3 ml water, three times extracted with 5 ml portions of ether, dialysed and lyophylised.

Hydrolysis. Samples of protein (50 mg) were hydrolysed in 5 ml twice-distilled 6 *M* HCl in evacuated vials at 110° for 32 h. The excess HCl was evaporated in a desiccator over KOH. The residue was twice taken up in water, filtered if necessary, again evaporated *in vacuo* and finally dissolved in 2.5 ml 10 % isopropanol.

Paper chromatography was carried out on Whatman paper No. 1. Histidine and tyrosine were identified by ascending–descending chromatography[13] (solvent butanol–2 *N* NH_3) and diazotized sulphanilic acid.

Paper electrophoresis of insulin was carried out on Whatman paper No. 3 MM as described previously[6].

Benzimidazole and benzimidazole N-sulphonic acid, prepared according to Weidenhagen[14] were run on Whatman paper No. 1 at 4.5 V/cm for 5 h and located by their ability to absorb u.v. light[15]. Benzimidazole had travelled 85 mm to the cathode, the sulphonic acid 3 mm to the anode.

Staining of insulin derivatives. Electropherograms of preparation I were the only ones that could be stained by bromophenol blue-$HgCl_2$ or amido black. The chlorine–tolidine reaction as described by Reindel[16] was therefore applied with one modification: after the stain had developed the strips were not washed in 2 % acetic acid, as recommended by Reindel, but in distilled water. This improved the stability of the colour sufficiently to allow the strips to be photographed without haste.

Biological assays were carried out on groups of 6 rabbits. The injection levels were a factor of 2 to 3 apart. The values recorded in Fig. 1 are considered to be accurate to approx. 20 %. No protracted action was observed.

Amino nitrogen analyses were carried out in threefold on 10 mg samples, according to Van Slyke's manometric method[17]. A solution of 6 % mercuric acetate in 2 %

acetic acid was applied, in a manner and for the purpose described previously[6]. Reaction period was 15 min at 22–23°. Strong light on the reaction chamber was avoided by the use of black paper.

The results are presented in Table III. Preparations III, IV and V had low and equal values. It was assumed that all amino groups had been converted and that the low values were only seeming ones and due to unknown side reactions. A correction was applied accordingly in calculating the number of free amino groups as presented in Fig. 1.

TABLE III

ANALYTICAL DATA

Preparation no.	*Reagent g/g insulin*	*pH*	*N %*	*S %*	*S/65 N*	$\Delta E^{1\,cm}_{1\%}$	*% NH_2*	*Biological activity*
Native insulin*	—	—	15.5	3.30	6.0^{5}**	15.6^{5}	0.77***	26.5 I.U.
I	0.1	10.0	13.9	3.88	7.9	13.5	0.36^{4}	11 I.U.
II	0.2	10.0	13.5^{5}	4.80	10.2	10.3^{5}	0.14	8 I.U.
III	0.5	10.0	13.4	5.35	11.3^{5}	8.5	0.07^{3}	4 I.U.
IV	1.0	10.0	13.3	6.01	12.8	3.9^{6}	0.06^{7}	1.5 I.U.
V	1.0	11.0	13.0	6.55	14.3 ± 0.2	0.6	0.07^{6}	

* Dried 16 h *in vacuo* at 100°.
** Calculated 6.00.
*** Calculated 0.76 %[6].

The assumption of complete sulphation of the amino groups was confirmed by HARDINGS's[18] ninhydrin method. Preparation V yielded only 3½ % of the colour of native insulin after a heating period of 3 min. This short heating period was chosen in order to limit hydrolytic fission of N–S and other N-bonds and precipitation of native insulin in the standard.

Further confirmation was obtained from the DNP derivative of preparation V. After hydrolysis no yellow colour (from DNP glycine and DNP phenylalanine) was observed in the ether extract and no ε-DNP lysine in the water layer (see page 105).

Free phenolic groups of tyrosine were determined from the difference in u.v. absorption at 295 mμ in 0.2 *N* NaOH and in neutral or weakly acid medium. The values observed are presented in column 7 of Table III in terms of

$$\Delta E^{1\,cm}_{1\%} = \left(E^{1\,cm}_{1\%}\right)\text{ alkaline} - \left(E^{1\,cm}_{1\%}\right)\text{ neutral}$$

where $E^{1\,cm}_{1\%}$ denotes the extinction of a 1 % solution of the preparation concerned in a 1 cm cuvet at 295 mμ. Since the absorption in alkaline medium slightly increased with time, it was measured as a function of time and extrapolated to zero time.

The absorption of native insulin was determined in a moist preparation and corrected for the moisture content. The value given in Table III corresponds to 3.95 tyrosine residues per insulin molecule, if the molecular extinction coefficient of tyrosine is taken as 2330[19] and the insulin contains 65 N atoms per molecule[5].

Histidine was determined by a combination of paper chromatography and diazo reaction[20]. The results recorded in Table IV are based upon N determinations in the hydrolysates.

As can be seen from this table, histidine is effectively removed by dinitrophenylation, since only 4 % of the initial content remains.

TABLE IV

HISTIDINE CONTENT OF INSULIN PREPARATIONS

Preparation	*Histidine* %
Native insulin	5.3*
DNP insulin	0.2
Insulin sulphate V	4.4[5]
DNP insulin sulphate V	3.1

* Calculated 5.4 %

The sulphated preparations proved to be resistant to hydrolysis. Even after 32 hours at 110° a small part of the samples was not dissolved in 6 *N* HCl and removed by filtration. This may explain why the recovery of histidine in preparation V is only 84 %, since those molecules which were dissolved shortly before the termination of the hydrolysis were only partly hydrolysed.

The histidine content of the DNP derivative of preparation V is 70 % of that of preparation V itself, *i.e.* 1.4 out of the 2.0 histidine residues were protected against DNFB by sulphation. This compares favourably with the 1.5 groups recorded in Fig. 1.

Total sulphur was determined by combustion in oxygen and conductometric titration[21].

Total nitrogen was determined by McKenzie's modification of the Kjeldahl method[22].

The data of Fig. 1, including the biological activities, are calculated from those of Table III, and are based upon the N content of the preparations, assuming 65 N atoms per insulin molecule (equal to 15.9 % N)[5].

The number of SO_3 groups introduced is equal to the total number of S atoms per molecule, minus 6.0. The data are accurate to ± 0.2 group. The accuracy of free amino and phenolic groups is about 0.1 group.

ACKNOWLEDGEMENT

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THE DENATURATION OF PEPSIN

IV. THE EFFECTS OF TEMPERATURE

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SUMMARY

The temperature coefficient of pepsin denaturation has been measured as a function of pH in solutions of neutral salt (KNO_3), guanidinium chloride and $Pb(NO_3)_2$. In KNO_3 the heat of activation has been found to decline uniformly by almost 50 kcal between pH 6.0 and 6.7. This is precisely the pH region where a number (5 or 6) of carboxyl groups, which appear to be hydrogen bonded in the native protein, are cleaved by a first order process when the enzyme is inactivated. In $6.6 \cdot 10^{-4}$ *M* $Pb(NO_3)_2$ solutions the $\Delta H^{\ddagger}$ *vs.* pH curve is displaced by about 0.4 pH units in an acid direction. Guanidine also diminishes the heat of activation. In 1.18 *M* guanidine, the heat of activation shows a similar decline above pH $\sim$6.0 as observed in KNO_3.

INTRODUCTION

In earlier reports potentiometric titration[1] and inactivation rate data[2] have been presented from which it has been concluded that about 5 or 6 carboxyl groups are hydrogen bonded in pepsin. Moreover, these groups are thought of as forming an integral part of the network of secondary valence structures which are responsible for maintaining the enzyme in its active state. However, it was also shown that the environmental conditions which determine the pH stability of pepsin could be modified by either hydrogen bond-breaking reagents, such as urea or guanidine or by small amounts of ions possessing a high affinity for either the proton or the carboxylate anion of the hydrogen bonded carboxyl groups.

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It is the purpose of this paper to explore the temperature coefficients of pepsin denaturation when reagents are present which are known to accelerate the rate of inactivation by modifying the structure of the carboxyl hydrogen bonds.

METHODS AND MATERIALS

The methods used and the sources of materials have been reported in previous communications in this series[1–3].

Inactivation experiments, conducted at a series of temperatures, were generally performed in unbuffered solutions of pepsin in water containing at least 0.15 M KNO_3 to fix the ionic strength. The release of protons that occurred during denaturation was monitored and compensated for by a manually operated pH-stat arrangement with a Beckman Model GS pH meter. The pH meter was calibrated with phosphate buffers at the same temperature as the inactivation experiment. All pH values listed were measured at the temperature of the experiment, except where otherwise stated.

Pepsin activities were determined by enzyme assay using hemoglobin as substrate. A few experiments were performed in phosphate buffer, where rates were measured by both enzyme assay and a solubility method. All rates were first order. Heats of activation were evaluated from the slope of a plot of the logarithm of the first order velocity constant *vs.* the reciprocal absolute temperature.

A solubility method was developed, which was quite similar to that used by NORTHROP[4]. The procedure consisted of precipitating denatured pepsin with a mixture of 10% trichloracetic acid, 0.2 M HCl and 1.0 M NaCl. The precipitate was separated immediately by centrifugation in a low speed clinical centrifuge. The O.D. of the supernatant was measured at 280 mμ. This method gave results that were identical to the hemoglobin assay at pH 6.65 and 7.00. At lower pH values and higher temperatures, denatured pepsin is more subject to hydrolysis by active pepsin and would complicate this assay. If we, therefore, define denaturation, in its most common usage, as the loss of solubility (at the isoelectric point) then we can speak of the denaturation or inactivation of pepsin interchangeably, since both processes proceed at identical rates.

Constant experimental temperature was maintained by circulating water from a constant temperature bath through a water-jacketed beaker which served as the titration vessel. The temperatures quoted are those of the solutions in the beaker, which differed slightly from that in the bath when working at temperatures other than that of the room.

RESULTS

The temperature coefficient of inactivation rates

KNO_3: One of the most characteristic features of protein denaturation reactions is their unusually high temperature coefficients. In this respect, pepsin denaturation is quite typical. Activation energies (E) may be calculated from the dependence of reactions on temperature by the Arrhenius equation. The heat of activation ($\Delta H^{\ddagger}$) is readily obtained[5] from the energy of activation by $\Delta H^{\ddagger} = E - RT$. Since RT is generally small compared with E, we have equated E with $\Delta H^{\ddagger}$.

Free energies of activation ($\Delta F^{\ddagger}$) were obtained from the relation $k = k'T/h$

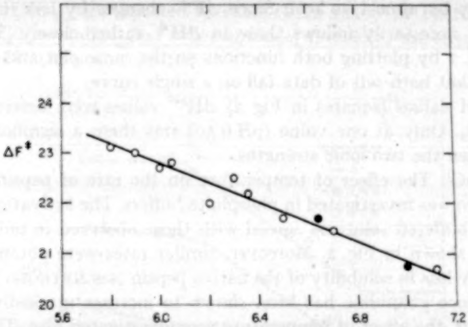

Fig. 1. Variation of the free energy of activation ($\Delta F^{\ddagger}$) with pH. ○, 0.15 *M* KNO_3; ●, 0.15 *M* NaCl–0.05 *M* phosphate.

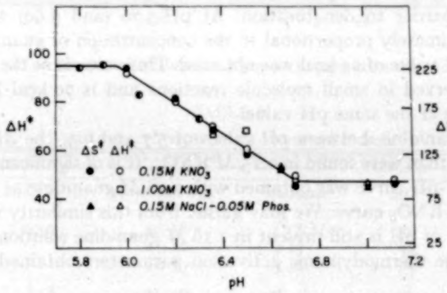

Fig. 2. Variation of the heat ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) of activation of pepsin denaturation with pH.

$e^{-\Delta F^{\ddagger}/RT}$ and entropies of activation ($\Delta S^{\ddagger}$) from $\Delta F^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$, where *k* is the first order rate constant in seconds, *k'* is the Boltzman constant, *T*, the absolute temperature, *h*, the Planck constant and *R* the gas constant. Thermodynamic activation parameters have been computed and appear in Figs. 1 and 2*.

Since the inactivation of pepsin goes to completion under all conditions presently investigated, only activation parameters can be obtained. The free energy of activation was found to decrease linearly with pH throughout the range investigated, *i.e.*, pH 5.80 to 7.15. This decline in $\Delta F^{\ddagger}$ reflects the rapid increase in rate that occurs in this pH zone. In contrast to the monotonic change in $\Delta F^{\ddagger}$, the variation of the heat (and entropy) of activation with pH (Fig. 2) shows two pH independent zones, one below 6.0 and one above 6.7, with a transition region between these values wherein

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* The reported values of $\Delta F^{\ddagger}$ in both the Figures and the Tables are average values collected over a temperature range indicated in the Tables. Actually the values of $\Delta F^{\ddagger}$ increased as the temperature decreased-usually about 1 kcal over the temperature range investigated. Since the predominant effects are observed in $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, which presumably do not vary with temperature, only average values of $\Delta F^{\ddagger}$ have been reproduced.

$\Delta H^{\ddagger}$ falls linearly by almost 50 kcal. Since $\Delta F^{\ddagger}$ changes by less than 3 kcal the variation in $\Delta S^{\ddagger}$ necessarily follows those in $\Delta H^{\ddagger}$ rather closely. This is shown graphically in Fig. 2 by plotting both functions on the same plot and adjusting the ordinate scale so that both sets of data fall on a single curve.

At several pH values (squares in Fig. 2) $\Delta H^{\ddagger}$ values were determined in 0.15 and 1.00 *M* KNO_3. Only at one value (pH 6.50) was there a significant difference (~ 10 kcal) between the two ionic strengths.

Phosphate–NaCl: The effect of temperature on the rate of pepsin inactivation at pH 6.65 and 7.00 was investigated in phosphate buffers. The activation parameters obtained in these buffered solutions agreed with those observed in unbuffered solutions of KNO_3 as shown in Fig. 2. Moreover, similar rates were obtained by either enzyme assay or by loss in solubility of the native pepsin (see METHODS Section).

Guanidine: Since guanidine has been shown to increase markedly the rate of pepsin inactivation[2], the effect of temperature was investigated also. The concentration of KNO_3 was increased to 0.50 *M* in this series of experiments. Some typical data are illustrated in Fig. 3. The data, in terms of activation constants, are summarized in Table I and Fig. 4. At all pH values studied, guanidine served to decrease the thermal energy barrier to denaturation. At pH 5.70 (and 5.90) the reduction in $\Delta H^{\ddagger}$ was approximately proportional to the concentration of guanidine. In 3.18 *M* guanidine, a $\Delta H^{\ddagger}$ value of 25 kcal was obtained. This value is in the range of activation energies observed in small molecule reactions and is 70 kcal less than occurs without guanidine at the same pH value.

In 1.18 *M* guanidine, between pH values of 5.7 and 6.5, the $\Delta H^{\ddagger}$ values were about 25 kcal less than were found in 0.15 *M* KNO_3. It is of significance that a similar break in the $\Delta H^{\ddagger}$-pH curve was obtained with 1.18 *M* guanidine as was observed in the corresponding KNO_3 curve. We may gather from this similarity in behavior that the intrinsic effect of pH is still present in 1.18 *M* guanidine solutions.

$Pb(NO_3)_2$: The thermodynamic activation parameters obtained in solutions of

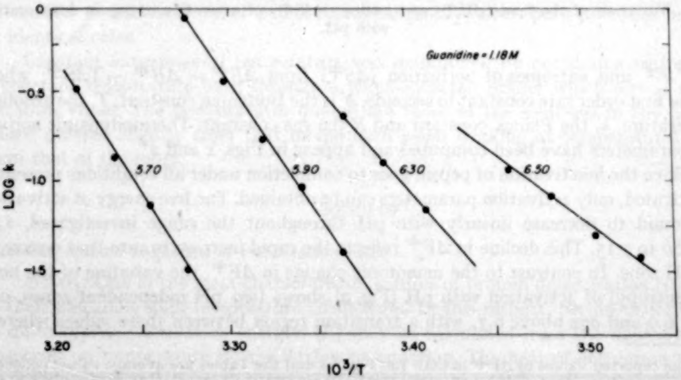

Fig. 3. The effect of pH on the temperature coefficient of pepsin inactivation in 1.18 *M* guanidine solutions. Pepsin concentration ≃ 0.2 %; KNO_3 = 0.50 *M*. *k* is the first order velocity constant in reciprocal minutes.

$6.6 \cdot 10^{-4}$ *M* $Pb(NO_3)_2$ are reported in Table II. In $1 \cdot 10^{-3}$ *M* $Pb(NO_3)_2$ at pH 5.9 the inactivation did not follow first order kinetics; therefore, the effect of Pb^{++} concentration on $\Delta H^{\ddagger}$ was not evaluated.

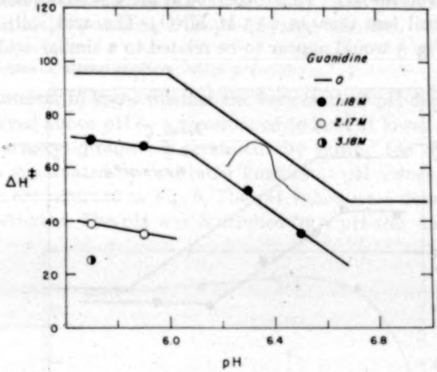

Fig. 4. Influence of pH on the heat of activation of pepsin in guanidine solutions. KNO_3 = 0.50 *M*.

TABLE I

THE EFFECT OF pH AND GUANIDINE ON THE ACTIVATION PARAMETERS OF PEPSIN DENATURATION*

pH	*KNO₃*	*Temperature range*	*Guanidine*	*ΔH‡*	*ΔS‡*	*ΔF‡*
5.70	0.50	32–38°	1.18 *M*	71	160	21.9
		21–26	2.17	39	58	21.5
		10–17	3.18	25	16	20.7
5.90	7.15	41–49	0	95	226	23.0
	0.50	24–34	1.18	68	156	21.0
		13–19	2.17	35	50	20.6
6.30	7.15	31–37	0	73	164	22.5
	0.50	20–24	1.18	52	105	20.9
6.50	0.15	24–33	0	60	125	21.7
	0.50	10–16	1.18	35	49	21.1

* $\Delta F^{\ddagger}$ and $\Delta H^{\ddagger}$ in kcal/mole; $\Delta S^{\ddagger}$ in cal/mole/degree.

TABLE II

THE EFFECT OF pH ON THE ACTIVATION PARAMETERS OF PEPSIN DENATURATION*

pH	*Temperature range*	*ΔH‡*	*ΔS‡*	*ΔF‡*
5.80	37–44°	74	164	22.8
5.90	32–36	76	176	22.0
6.00	30–36	67	148	21.8
6.20	22–30	57	120	21.5
6.40	18–30	40	64	21.1
6.60	14–22	42	75	20.7

* In $6.6 \cdot 10^{-4}$ *M* $Pb(NO_3)_2$ (KNO_3 = 0.15 *M*).

The heats of activation are plotted as a function of pH in Fig. 5. where they may be compared with the corresponding values found in 0.15 *M* KNO_3. The two curves are similar in shape, with the Pb^{++} curve being displaced by about 0.4 pH units in an acid direction. However, the $\Delta H^{\ddagger}$ value observed at pH 5.7 did not fit on the displaced curve and was 20 kcal less than in 0.15 *M* KNO_3. The acid shift evident in the $\Delta H^{\ddagger}$-pH curve of Fig. 5 would appear to be related to a similar acid shift observed in the irreversible segment of pepsin "titration curves" when conducted with Pb^{++} present[1]. When Zn^{++} was added at 7.5 times as large a concentration as Pb^{++}, a value of 65 kcal was found for $\Delta H^{\ddagger}$ at pH 6.4. This coincides with the value observed in 0.15 *M* KNO_3 solutions. This result is compatible with the much smaller effect of Zn^{++} that was observed on the rate of pepsin inactivation[2].

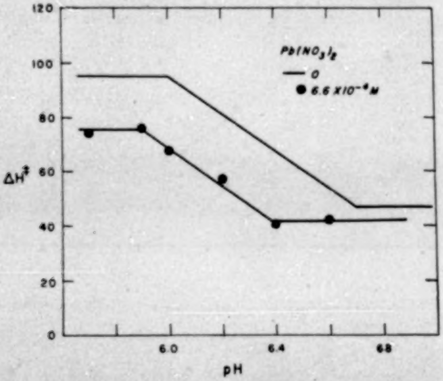

Fig. 5. Influence of pH on the heat of activation of pepsin in $6.6 \cdot 10^{-4}$ *M*. $Pb(NO_3)_2$. KNO_3 = 0.15 *M*.

Ethyl alcohol: It has been established that the rate of pepsin inactivation increases with the 4.5th power of the molar concentration of ethyl alcohol[2]. Moreover, the pH dependence of inactivation remains constant between 0 and 38 % (v/v) ethyl

TABLE III

THE EFFECT OF ETHYL ALCOHOL ON THE ACTIVATION PARAMETERS OF PEPSIN* AND HEMOGLOBIN** DENATURATION

pH	*Temperature range*	C_2H_5OH	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta F^{\ddagger}$
5.90*	41–49	0	95	226	23.0
	31–36	20 % (v/v)	88	215	22.0
	21–29	40 %	52	104	21.2
6.00–7.00**		0	76	153	25.1
		20 %	107	264	23.5
		30 %	117	309	22.5

* KNO_3 = 0.15 *M*.

** Hemoglobin data taken from work of BOOTH, ref.[18]. The activation parameters were similar between pH 6.00 and 7.00.

alcohol. It is assumed, therefore, that the mechanism of inactivation is unchanged in this range of alcohol concentration.

The kinetic activation constants in 20 and 40 % alcohol are included in Table III. In 20 % (v/v) alcohol the heat of activation is only slightly reduced from its value in water. However, in 40% alcohol, a drop of 43 kcal occurred.

The pH dependence of denaturation below pH 5.0

It was of interest to know whether the very marked pH dependence of inactivation rates observed above pH ~ 5.5 continued to hold at lower pH values where the ionization of carboxyl groups are repressed. By raising the temperature to above 60° pepsin was inactivated by first order kinetics at pH values between pH 3.4 and 5.0. These data are recorded in Fig. 6. The pH values were determined at 25° at the end of the experiment. The pH was controlled by a pH-stat during the experiment.

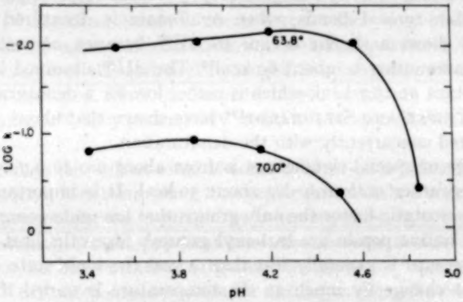

Fig. 6. Effect of pH on the first order velocity constant of pepsin inactivation at low pH values and high temperatures. *k* in reciprocal minutes.

It is apparent from Fig. 6 that between pH 3.4 and 4.6, the pH dependence is strikingly different from that found at higher pH values. In fact, the two curves illustrated in Fig. 6 show that the rate of inactivation first decreases slightly with increase in pH, then passes through a minimum in rate and finally increases very rapidly to give the exceptional pH dependence observed in the neutral range. It is interesting to note, that the heats of activation between pH 3.4 and 4.6 are high and agree approximately with those found between pH 5.7 and 6.0 (see Fig. 2). It may, therefore, be concluded that denaturation and not autolysis constitutes the principal pathway of inactivation between pH 3.4 and 4.6 at elevated temperatures.

DISCUSSION

Relationship to STEINHARDT'*s data*

A discrepancy appears to exist between STEINHARDT's temperature data and those reported presently[6]. STEINHARDT's value of the activation energy of pepsin denaturation was obtained from the difference in rates at only two temperatures, *i.e.*, 25° and 15°. The pH dependence was linear (inverse fifth order) up to a rate of about $\log k = -2$ (min^{-1}); the $\Delta H^{\ddagger}$ was considered constant and equal to 63.5 kcal/mole, from pH 6.1 to 6.4, the range of overlap of his two curves. The results reported in

Fig. 2 show a decline from about 80 to 65 kcal in this pH interval. Over the larger pH range shown in Fig. 2, the $\Delta H^{\ddagger}$ was observed to fall gradually from about 95 to 48 kcal between pH 6.0 and 6.7 and then remain constant. A reduction in $\Delta H^{\ddagger}$ above pH 6.4 is evident also in STEINHARDT's data[6].

Various values for the pH dependence of pepsin inactivation rates have appeared in the literature[6]. Since the temperature coefficient is known to vary with pH, this effect would directly lead to different values for the variation of rate with pH. Thus the pH dependence would tend to decrease as the reaction temperature was increased.

The variation of $\Delta H^{\ddagger}$ with pH

There are several examples of protein denaturation in the literature where the activation energy is rather strongly dependent on pH. Thus there is a decrease of about 100 kcal in $\Delta H^{\ddagger}$ for ovalbumin when going from[7] pH 5.5–7.7 to[8] pH 1.0–1.7. HARRINGTON has shown recently that prototropic changes occur, apparently involving about 8-carboxylate-tyrosyl bonds, when ovalbumin is denatured in guanidine[9]. Hemoglobin also shows a similar decline in $\Delta H^{\ddagger}$ between neutrality and mildly acidic solutions, amounting to about 60 kcal[10]. The $\Delta H^{\ddagger}$ observed between pH 3.2 and 4.1 was constant at 15.7 kcal, which is rather low for a denaturation reaction[11]. In this pH zone ZAISER AND STEINHARDT[12][*] have shown that about 36 acid-binding groups are liberated concurrently with the denaturation.

The pH range of special significance is from about 6.0 to 6.7, where the heat of activation falls rather uniformly by about 50 kcal. It is important to note that due to a large electrostatic factor the only groups that are undergoing dissociation in this pH region in native pepsin are carboxyl groups[1]. Since the heat of dissociation of free carboxyl groups is generally less than 2 kcal the ionic state (net charge) of pepsin should not change by much as the temperature is varied if the pH, ionic strength and other variables are kept constant. The observed heats of activation, therefore, would not include a significant contribution from their heat of ionization. Of the 63.5 kcal activation energy STEINHARDT attributed about 45 kcal to the heat of ionization of 5 amino groups which were assumed to be the critical charged groups involved in pepsin denaturation[6]. Since the critical groups have been shown to be carboxyl[1], the observed values of $\Delta H^{\ddagger}$ would, therefore, represent the thermal energy required to activate weak, noncovalent bonds in pepsin. Consequently, the decrease in $\Delta H^{\ddagger}$ found between pH 6.0 and 6.7 should be related to the activation of hydrogen-bonded carboxyl groups. On this basis, these groups require almost 50 kcal of activation energy. Above pH 6.7 where $\Delta H^{\ddagger}$ appears to be invariant with pH again only the remaining weak bonds (*i.e.*, α-helical, hydrophobic, etc.) need to be activated and they absorb about the same amount of thermal energy.

Below pH ∼ 5.5 only free carboxyl groups are dissociated and the rate of pepsin inactivation shows but a small dependence on pH. Above pH ∼ 5.5. the more stable hydrogen-bonded groups tend to ionize and the rate of inactivation becomes strikingly dependent on pH. With perturbations of these bonds resulting from the lower acidity of the medium, their dimensions would tend to increase and their strength to diminish. An increase in entropy would also result. The reduction in $\Delta H^{\ddagger}$ that occurs above pH 6.0 indicates that the ionizing tendency of hydrogen-bonded groups can facilitate their activation. The parallel decline that takes place in $\Delta S^{\ddagger}$ between pH 6.0 and 6.7 reflects a greater entropy of the initial state of the molecule provided that the structure of the activated state does not vary much with the small increase in pH.

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[*] In a recent report S. BEYCHOK AND J. STEINHARDT (abstracts of Papers, Am. Chem. Soc., Chicago, Ill. Sept. 7–12, 1958, p. 41C.) claim that 20% of these groups are due to electrostatic factors and 80% to unreactive acid binding groups.

In an earlier paper[1] it was suggested that the unusually large pH dependence of inactivation rates implied an acceptor group (to the carboxyl donor) which possessed a p*K* quite close to that of the donor. On this basis, we proposed that the acceptor group was also a carboxyl group which formed a double hydrogen bond with the donor group. The marked pH dependence of $\Delta H^{\ddagger}$ should provide additional support for this hypothesis. It is of interest consequently to try to correlate that part of the activation energy associated with this bond with the heat of the carboxyl–carboxyl hydrogen bond. In acetic acid, the energy of this homologous double bond is close to 14 kcal[13]. If three such bonds occur in pepsin, which is compatible with the titration data, a ΔH value of 42 kcal is obtained. The difference between this value and that of $\Delta H^{\ddagger}$ for these bonds is about 5 kcal which may readily be accounted for by a small positive heat of ionization, *i.e.*, ~1 kcal per bond. The utilization of the full energy value of the carboxyl hydrogen bond assumes that these bonds are not significantly solvated. Since carboxyl hydrogen bonds are not very stable in an aqueous environment[14], they presumably would not be appreciably solvated in native pepsin.

Since guanidine (as well as urea) is considered to be a better hydrogen-bonding solvent than water, it is not surprising that pepsin is more labile in guanidine solutions. The increase in rate observed in guanidine is accompanied by a reduction in heat of activation (see Fig. 4). At pH 5.70 (and 5.90), the decrease in $\Delta H^{\ddagger}$ is approximately proportional to the concentration of guanidine. MIHALYI[15] has reported a similar decline in $\Delta H^{\ddagger}$ with urea concentration for fibrinogen denaturation. Since guanidine can reduce $\Delta H^{\ddagger}$ below the values observed in aqueous media, it seems likely that hydrogen-bonded groups in pepsin other than carboxyl are activated also by guanidine.

It has been demonstrated elsewhere that Pb^{++} (and other metals having a large affinity for carboxylate groups) can increase the acid strength of the hydrogen-bonded groups in pepsin[1]. By enhancing the tendency of these groups to ionize, and consequently to rupture hydrogen bonds, the thermal energy required for activation is decreased. The ability of Pb^{++} to reduce $\Delta H^{\ddagger}$ is essentially no greater that that accomplished by lowering the acidity of the solution, since both the Pb^{++} and KNO_3 curves reach a limiting value of 44 ± 3 kcal for $\Delta H^{\ddagger}$ and become independent of pH at higher values. The effect of Pb^{++} upon $\Delta H^{\ddagger}$ would therefore, be restricted to that part of $\Delta H^{\ddagger}$ required to activate carboxyl bonded groups.

It is instructive to compare the effect of ethyl alcohol on the kinetic behavior of pepsin with that of hemoglobin[8, 16]. The rate of denaturation of both proteins is increased as alcohol is added to the aqueous protein solutions. However, the heat of activation increases with hemoglobin and decreases with pepsin as the concentration of ethyl alcohol increases. Since the reduction in the free energy of activation that occurs upon the addition of ethyl alcohol is fairly small, the entropy of activation must increase considerably for hemoglobin denaturation and accordingly decrease

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[*] It is noteworthy that a larger entropy of activation (~135 e.u.) is associated with the activation of the carboxyl hydrogen-bonded groups than with the residual weak interactions where an increase of only 90 e.u. was calculated.

for pepsin inactivation (see Table III). The difference in $\Delta H^{\ddagger}$ between water and a 40 % ethyl alcohol solution of pepsin is about 43 kcal. A 30 % alcohol solution produces an increase in $\Delta H^{\ddagger}$ of about 40 kcal for hemoglobin denaturation[16]. Both changes in $\Delta H^{\ddagger}$ occur at pH values where $\Delta H^{\ddagger}$ in water is close to a maximum.

In solutions where the activity of water is reduced, as in water–alcohol mixtures, the solvent should be less effective than water in disrupting the hydrogen bonding in a protein solute. On the other hand, intramolecular bonding between hydrophobic side chain residues will tend to be less stable than in water due to the stronger solvating ability of the non-polar chain of the alcohol. It would appear, therefore, that the influence of alcohol on $\Delta H^{\ddagger}$ would depend on the relative contributions of the hydrogen and hydrophobic bonding situations to the intramolecular cohesive energy. If the 36 (or 18 per 34,000 molecular weight) acid binding groups liberated in hemoglobin denaturation are hydrogen-bonded in the native protein, these bonds would provide a major source of stability. This situation is to be contrasted with the relatively small number of carboxyl hydrogen bonds (5 or 6) in pepsin (M = 36,000). From the effect of alcohol on $\Delta H^{\ddagger}$, we may surmise that hydrophobic bonds provide a larger stabilizing force in pepsin than in hemoglobin. In this connection, it is interesting to note that the heat of activation for hemoglobin denaturation, in acid, is only 16 kcal, whereas in pepsin denaturation, above pH 6.7, it is still close to 50 kcal. The large increase in $\Delta S^{\ddagger}$ for hemoglobin denaturation in 30 % alcohol is suggestive of a much more ordered structure for native hemoglobin in this solvent. This could occur by more extensive hydrogen bonding, especially in the secondary structure (peptide hydrogen bonds), resulting from the rearrangement (or reduction) in the hydrophobic interactions.

ACKNOWLEDGEMENTS

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PAPER CHROMATOGRAPHY OF HEPARIN AND RELATED SULFATED MUCOPOLYSACCHARIDES*

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SUMMARY

A paper chromatographic system was developed for the resolution of mixtures of sulfated mucopolysaccharides. The effect of varying the ammonium formate buffer/isopropanol ratio in the developing solution on the R_F values of heparin and chondroitin sulfate is presented.

A complete separation of heparin from chondroitin sulfate was obtained with three solvent mixtures containing different proportions of buffer and isopropanol.

One of these solvent mixtures, ammonium formate buffer–isopropanol (65:35) v/v, resolved commercial beef heparin into two principal components, on "fresh" paper. This system was used to compare beef heparin with mouse mast cell tumor heparin, rat heparin, sheep lung heparin, chondroitin sulfate, and β-heparin. A common metachromatic component with an R_F of approximately 0.5 was found in all of the heparin preparations chromatographed with this solvent mixture.

"Aging" of the filter paper resulted in the loss of the ability of the buffer–isopropanol mixture (65:35) v/v to resolve the beef heparin. Increase of the buffer–isopropanol ratio to (70:30) v/v restored the resolution of the beef heparin on "aged" paper. Heparitin sulfate and α-heparin monosulfate were compared to beef heparin using the latter system.

INTRODUCTION

A procedure for the extraction of heparin from tissues was reported recently from this laboratory[1]. For a study of heparin biosynthesis in a mouse mast cell tumor[2-4], it was necessary further to purify the heparin preparation obtained by this technique. Filter paper chromatography appeared to be the most desirable method for separating the heparin from other sulfated mucopolysaccharides, in particular, chondroitin sulfate.

A search of the literature revealed only two reports[5,6] in which a separation of heparin from chondroitin sulfate was accomplished by paper chromatography. Two solvent systems were used to effect this separation. When the mixture ethanol–phosphate buffer was used, heparin remained essentially on the origin, while chondroitin sulfate moved close to the solvent front. With the other solvent system

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[*] Presented, in part, at the 49th annual meeting of the American Society of Biological Chemists in Philadelphia, April 1958.

described, *n*-propanol–phosphate buffer, heparin was separated from chondroitin sulfate, but both migrated close to the solvent front, and close to one another. It appears that in order to establish chondroitin sulfate contamination of a heparin preparation, both of these solvent systems must be used. In addition, the patterns obtained, while adequate for a characterization of the chondroitin sulfate, were not considered satisfactory for a characterization of the heparin. Other published reports[7,8] of the filter paper chromatography of heparin do not adequately describe the conditions used, nor, as mentioned above, is the behavior of chondroitin sulfate in these systems indicated.

An attempt was made, therefore, to develop a relatively simple and more convenient paper chromatographic technique for the separation of heparin from closely related compounds. Using a solvent system consisting of buffer at a lower pH than previously employed, and isopropanol, the effects of varying the buffer/alcohol ratio of the developing solution on the chromatographic behavior of heparin and chondroitin sulfate were investigated. The results of these experiments, and a paper chromatographic technique for the separation of heparin from related compounds developed in the course of this study, are presented below.

Procedure

Using an aqueous solution containing 6 mg of a single mucopolysaccharide/ml, a quantity of 30 µg of material* was applied (spot diameter, 7–10 mm) to a strip of Whatman No. 1 filter paper, 4¼ in. × 16 in., at a distance of 3 in. from the end of the strip. Spots containing a mixture of mucopolysaccharides contained 30 µg of each substance.

In general, apparatus standard for descending filter paper chromatography was employed. The atmosphere of a chromatographic jar (6 in. × 18 in.) was equilibrated overnight with the solvent to be used. The spotted paper was then placed in the jar. An anti-siphon rod was in position 1.5 in. from the spots, and 1.5 in. from the end of the paper, which was held in a glass trough. After equilibration of the paper with the atmosphere of the jar for 3–4 h, the solvent was introduced into the trough. The chromatogram was developed for about 12 h at room temperature (26 ± 2°). The paper was then removed from the jar, the solvent front marked with a pencil, and the paper dried at room temperature. The results shown in Fig. 1 were obtained in this manner.

In order to obtain a longer distance from origin to solvent front, a chromatocab (16.5 in. wide, 27 in. long, 25.5 in. deep) was used in some instances. Whatman No. 1 filter paper strips, 22 in. long, were used. The width of these strips depended on the number of individual samples to be chromatographed simultaneously. Materials were applied 4 in. from one end of the paper. An anti-siphon rod was in position 1.5 in. from the applied spots, and 2.5 in. from the end of the paper which was held in the glass trough. The time of development was approx. 16 h. Other details of technique were described above for the jar system. The results represented in Figs. 2–4 were obtained in the chromatocab.

The solvents used to develop the chromatograms consisted of a mixture of 0.04 *M*

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* In the case of the mouse mast cell tumor heparin, three units were applied (assay according to U.S.P. XIV).

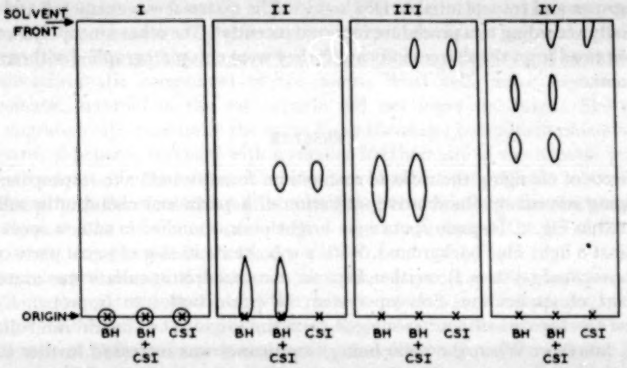

Fig. 1. Paper chromatography of commercial beef heparin (BH) and chondroitin sulfate CSI. Solvent systems consist of 0.04 *M* ammonium formate buffer, pH 4.3, and isopropanol in the following proportions; I: 50:50 (v/v); II: 55:45 (v/v); III: 60:40 (v/v); IV: 65:35 (v/v).

TABLE I

COMPOSITION OF SOLVENT SYSTEMS USED

Buffer: 0.04 *M* ammonium formate (General Chemical Division, Allied Chemical and Dye Corp., Reagent) adjusted to pH 4.3 with formic acid (Mallinckrodt Chemical Works, A. R., 88%). Isopropanol (General Chemical Division, Allied Chemical and Dye Corp., Reagent, A.C.S.).

Solvent system	*Parts buffer (v)*	*Parts isopropanol (v)*
I	50	50
II	55	45
III	60	40
IV	65	35
V	70	30

ammonium formate buffer, pH 4.3, and isopropanol, in varying proportions, as shown in Table I.

Dried chromatograms were stained by immersion in an Azure A solution for a period of 4 to 5 min. This solution was prepared by dissolving 200 mg of Azure A (National Aniline Division, Allied Chemical and Dye Corp., certified, 93 % dye) in 50 ml distilled water, and then adding 400 ml acetone (General Chemical Division, Allied Chemical and Dye Corp., Reagent, A.C.S.), and 1800 ml methanol (General Chemical Division, Allied Chemical and Dye Corp., Reagent, A.C.S.) to this solution. Upon removal from the staining solution, the paper was dried in air at room temperature.

Some of the Whatman No. 1 filter paper strips were used relatively soon after having been purchased; this paper was designated "fresh". In other instances, the paper strips were stored in a closed, but not airtight, container for approximately one year; these strips were designated "aged".

The mouse mast cell tumor heparin was prepared from Dunn-Potter tumor[9] tissue grown and transplanted in Dba/2 mice. The material was extracted and purified essentially according to a procedure reported recently[1]. The other mucopolysaccharides were obtained from the sources indicated; they were chromatographed without further treatment.

RESULTS

The effects of changing the ratio of ammonium formate buffer to isopropanol in the developing solvent on the relative migration of heparin and chondroitin sulfate are presented in Fig. 1. Heparin spots were bright pink, chondroitin sulfate spots purple, all against a light blue background. With a solvent consisting of equal parts of buffer and isopropanol (system I), neither heparin nor chondroitin sulfate was moved from the point of application. Solvent system II (ratio buffer to isopropanol, 55:45) streaked the heparin without moving it from the origin. The chondroitin sulfate was shifted, however. When the ratio buffer/isopropanol was increased further to 60:40 (system III), both heparin and chondroitin sulfate migrated from the origin, and a complete separation of these compounds was accomplished. System IV, with a ratio of 65:35, resolved heparin into two metachromatic spots, a slower moving major component, and a faster moving minor component. Chondroitin sulfate moved with the solvent front in this system.

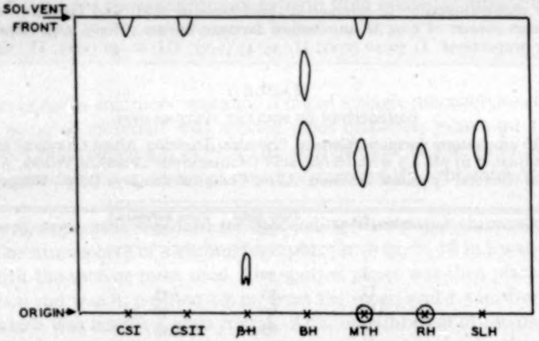

Fig. 2. Paper chromatography of chondroitin sulfate of different source (CS I, CS II), of β-heparin (β-H), and of heparin of different origin (beef, BH; mouse tumor, MTH; rat, RH; sheep lung, SLH). Solvent system IV.

Solvent system IV was used to compare commercial beef heparin with mouse mast cell tumor heparin, rat heparin, sheep lung heparin, β-heparin*, and chondroitin sulfate** (Fig. 2). The spots represent pink or purple metachromasia with one exception: the point of application of the mouse mast cell tumor heparin stained a deep blue. The major metachromatic component of the mouse mast cell tumor heparin migrated in this system with an R_F slightly less than that of the major component of the commercial beef heparin; a smaller amount of metachromatic material moved with the solvent front. The rat heparin traveled with an R_F essentially equal to that of the major metachromatic component of the mouse mast cell tumor heparin. Some metachromatic material in the rat heparin did not leave the origin. Sheep lung heparin migrated with essentially the same R_F as the major component of commercial beef heparin. β-heparin traveled with a smaller R_F than any of the heparin preparations which were run in this system. Chondroitin sulfate moved with the solvent front. The components of the mouse mast cell tumor heparin and rat heparin which remained at the origin may have been responsible for a slight reduction of the R_F of the major metachromatic components of these preparations.

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* The term β-heparin was used, since this preparation was so designated by Dr. WINTERSTEIN, who furnished it. This compound is identical with chondroitin sulfate B [10].

** Two preparations of chondroitin sulfate (CS) were chromatographed: CS I purchased from Aldrich Chemical Co.; CS II supplied by Dr. WINTERSTEIN.

The prolonged storage of Whatman No. 1 filter paper strips, "aging", resulted in a change in the pattern obtained when beef heparin was chromatographed with solvent system IV: only a single spot with an R_F of about 0.4 was observed. However, when the ratio of buffer to isopropanol was increased to 70:30 (system V), the "aged" filter paper yielded essentially the same pattern for beef heparin as did "fresh" paper with solvent system IV (Fig. 3). "Aging" of the paper did not affect the R_F of chondroitin sulfate or of β-heparin when solvent system IV was used.

Using solvent system V, and "aged" paper, beef heparin was compared with α-heparin monosulfate and with heparitin sulfate (Fig. 4). The latter two substances stained red-violet. The spots were, however, much less intense than those which were obtained with an equivalent amount of beef heparin. α-heparin monosulfate migrated with an R_F essentially equal to that of the faster moving component of beef heparin. Heparitin sulfate yielded two spots corresponding to those of the commercial beef heparin used.

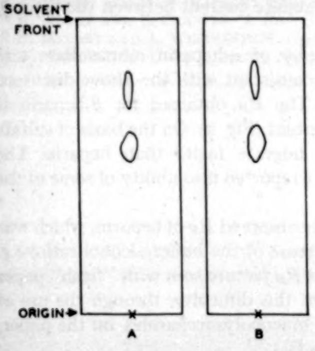

Fig. 3. Chromatography of commercial beef heparin. Comparison of "fresh" and "aged" paper. A: "Fresh" paper, solvent system IV; B: "Aged" paper, solvent system V.

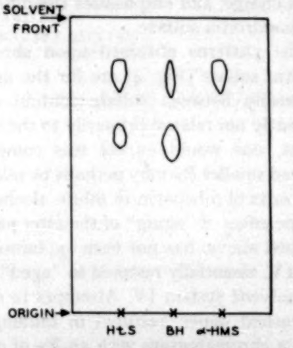

Fig. 4. Paper chromatography of heparitin sulfate (HtS), commercial beef heparin (BH), and α-heparin monosulfate (α-HMS); "aged" paper, solvent system V.

DISCUSSION

The purpose of this study was to develop a convenient chromatographic procedure which would permit the separation of heparin from chondroitin sulfate, and a characterization of the mouse mast cell tumor heparin. Solvent system IV, with "fresh" paper, appears to satisfy these requirements.

A complete separation of heparin from chondroitin sulfate was obtained with this system, with a greater difference in R_F values than was reported prior to this study. This large difference makes it much easier to evaluate the relative quantities of heparin and chondroitin sulfate in a mixture.

Furthermore, a much more satisfactory characterization of a heparin preparation is possible with this system, since the major metachromatic component of commercial beef heparin migrates with an R_F of 0.57. Sulfated mucopolysaccharides which conceivably could contaminate heparin preparations, such as chondroitin sulfate and β-heparin, migrate with R_F values markedly different from that of heparin (Fig. 2). The characterization of mouse mast cell tumor heparin was based, in part, on its chromatographic behavior with this solvent system[2-4].

The effects of the isopropanol concentration of the solvent system on the R_F values of heparin and chondroitin sulfate appear to be related to the relative non-polarity of the isopropanol. Both heparin and chondroitin sulfate, being highly polar molecules, remained at the origin when the isopropanol content of the developing solvent was too high (system I, Fig. 1). Heparin is more polar than chondroitin sulfate and was not moved from the origin by a solvent mixture (containing less isopropanol) which did move chondroitin sulfate (system II, Fig. 1)[*]. The polarity difference between heparin and chondroitin sulfate is again apparent from the differences in patterns obtained with solvent systems III and IV (Fig. 1).

The relatively low pH of the buffer reduces the ionization of the carboxyl groups of both the heparin and the chondroitin sulfate. Though this decreases the net negative charge on these molecules, it increases the relative contribution of the sulfate to this charge, and emphasizes the difference in sulfate content between the heparin and chondroitin sulfate.

The patterns obtained upon chromatography of α-heparin monosulfate and heparitin sulfate (Fig. 4) are for the most part consistent with the above-discussed relationship between sulfate content and R_F. The R_F obtained for β-heparin is apparently not related primarily to the sulfate content (Fig. 2). On the basis of sulfate content, one would expect this compound to migrate faster than heparin. The observed smaller R_F may perhaps be related to the reported insolubility of some of the metal salts of β-heparin in dilute alcohol[13-15].

The effect of "aging" of the filter paper on the observed R_F of heparin, which was described above, has not been explained. An increase of the buffer/alcohol ratio, *e.g.* system V, essentially restored to "aged" paper the R_F picture seen with "fresh" paper using solvent system IV. Attempts to circumvent this difficulty, through the use of acid-washed paper, resulted in binding of the mucopolysaccharides on the paper, giving a chromatogram with an R_F of essentially O.

When this study was initiated, we were not aware of a report by AWE AND STÜDEMANN[16] which presented three solvent systems for the paper chromatography of heparin, heparinoids, and "heparin-like" substances. However, separation of heparin and chondroitin sulfate was not obtained with any of the three solvent systems described by these authors.

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[*] Heparin has either 2.5 or 3 sulfate groups per disaccharide unit[11,12], as compared to only one [13] in the case of chondroitin sulfate.

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AN ELECTROPHORETIC STUDY ON STRUCTURAL COMPONENTS OF *MICROCOCCUS LYSODEIKTICUS*

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SUMMARY

Suspensions of intact bacteria, protoplasts, protoplast membranes and the derived membrane lipid and defatted membrane fractions of *Micrococcus lysodeikticus*, as well as cardiolipin, have been studied by micro-electrophoresis. The pH–mobility relationships and the influence on surface charge density of thorium and uranyl ions were used to characterize the ionogenic groups present on the surfaces. For this purpose the concept of isosteric ion adsorption concentrations is preferred in these systems to the use of ionic reversal of charge concentrations. For bacteria, protoplasts, protoplast membranes and defatted protoplast membranes, the electrophoretic behaviour is controlled by the ionization of carboxyl and amine groups in the surface, whilst the membrane lipid and cardiolipin are phosphate type colloids. The results provide experimental evidence to support the view that in the structure of the protoplast membrane, the lipid occupies an internal position sheathed by protein and possibly carbohydrate.

INTRODUCTION

Studies[1] on model systems consisting of known natural colloids have shown that each type of ionogenic group has a characteristic electrophoretic behaviour pattern and a characteristic sequence has been found for the concentrations of various ions required to reverse its charge, which is termed an "ion spectrum". In particular, thorium ions have been found to be more effective than uranyl ions in reversing the charge of carboxyl type colloids, but not for some phosphate types.

In recent years, micro-electrophoretic methods[2] have been used to investigate the electrokinetically active surface layers of several bacteria. In the present paper, the effects of hydrogen, uranyl and thorium ions on the electrophoretic properties of a number of components from *M. lysodeikticus* and its protoplasts are presented in order to characterize the types of ionogenic groups present in their surfaces and to provide information on protoplast membrane structure.

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METHODS

Materials

The organism used was *Micrococcus lysodeikticus* (N.C.T.C. 2665) grown on beef tryptic digest medium as in earlier work[3]. Electrophoretic measurements were made on suspensions of intact bacteria, protoplasts, protoplast membranes and the lipid and defatted membrane fractions derived from the protoplast membranes. The preparation of each of these has been described previously[3]. In addition, a sample of cardiolipin was kindly supplied by Dr. M. PANGBORN. Reagents were of A.R. quality.

Micro-electrophoresis

All measurements were made at 25° in a horizontal cylindrical glass micro-electrophoresis cell using methods previously described[4]. Most observations of particles were made under dark ground illumination. For any reading, ten observations were made, five in each direction, so as to minimise the effect of small random drifts.

Suspensions were prepared by shaking in 0.05 *M* NaCl. No buffer solutions were used because of possible specific ion interactions. To obtain pH-mobility relationships, the pH of each suspension was adjusted over the range 1 to 11 with small quantities of 0.05 *M* HCl and 0.05 *M* NaOH. Other measurements were made on suspensions in the presence of uranyl nitrate and thorium nitrate at a number of concentrations. Where necessary, the concentration of NaCl was reduced to maintain constant ionic strength. With the more concentrated uranyl nitrate solutions, the NaCl was replaced with NaOAc to reduce the lowering of pH produced by hydrolysis. Protoplasts had to be examined in the presence of isotonic sucrose (1 *M*) and, for comparison, some measurements on protoplast membranes were made in the same medium.

RESULTS

pH-mobility curves

The measured mobilities were converted to zeta potentials by means of the HELMHOLTZ-SMOLUCHOWSKI equation for large particles

$$\zeta = \frac{4\pi}{D}\,\eta\cdot v \qquad (1)$$

where η and D are the viscosity and the dielectric constant, within the electric double layer, assumed to be those for water, and v is the electrophoretic mobility. The pH of the surface at the plane of shear was then calculated from the equation of HARTLEY and ROE

$$\mathrm{pH_s} = \mathrm{pH_{Bulk}} + \frac{e\zeta}{2.303kT} \qquad (2)$$

where e is the electronic charge, k is the Boltzmann constant and T the absolute temperature.

In Fig. 1, the electrophoretic mobility has been plotted against $\mathrm{pH_s}$ for each system. The protoplasts and protoplast membranes showed identical behaviour in 1 *M* sucrose. Chiefly due to the effects of viscosity, the mobility values of membranes in 1 *M* sucrose, 0.05 *M* NaCl, were about one fifth of the corresponding mobilities in 0.05 *M* NaCl. It was concluded that protoplasts and the membranes derived from them possess similar electro-kinetic properties. The experimental difficulties arising from the lysis of protoplasts made it preferable to study protoplast membranes instead of intact protoplasts.

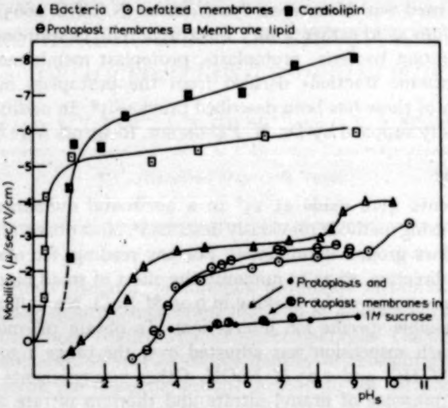

Fig. 1. pH$_s$-mobility curves of suspensions in 0.05 *M* NaCl of cardiolipin and bacterial derivatives.

p*K* values for the surface ionogenic groups have been derived from the curves in Fig. 1. The surface charge densities corresponding to the ionized and the un-ionized form of each group, revealed by the plateaux in the mobility–pH$_s$ curves, were calculated using the GOUY-CHAPMAN equation

$$\sigma = 3.53 \cdot 10^4 c^{\frac{1}{2}} \sin h \frac{e\zeta}{2kT} \tag{3}$$

where *c* is the concentration of uni-univalent electrolyte. Each mobility corresponding to the mean of the ionized and unionized charge densities was then estimated and the corresponding value of pH$_s$ obtained. The values of p*K* so derived are approximate because the surface ionogenic groups may not be precisely in the plane of shear. Further, where more than one type of charge group is present, the charge densities considered should strictly relate to conditions under which charge groups not under consideration are unionized.

Results are given in Table I. With bacteria and defatted protoplast membranes there appear to be both acidic and basic ionogenic groups in the surface. Protoplast

TABLE I

p*K* VALUES FROM ELECTROPHORETIC DATA ON CELL FRACTIONS

System	*pK$_A$*	*pK$_B$*
Bacteria	2.1	8.6
Protoplast membranes	3.6	—
Defatted membranes	3.6	9.95
Membrane lipid	0.4	—
Cardiolipin	1.05	—

membranes probably possess a basic group similar to the defatted membranes although, due to disruption of the membranes in alkaline media, it was not possible to demonstrate this experimentally. The membrane lipid is simpler in that it exhibits only one very acidic type of ionogenic group. For comparison, cardiolipin has been used as a purely phosphate type of colloid. The two lipids show a similar type of behaviour, although the membrane lipid has a significantly lower p*K*.

Charge groups and ion adsorption

The effects of the adsorption of uranyl ions and thorium ions on the colloid surfaces have been compared using surface charge densities calculated from mobility measurements by means of eqns. (1) and (3). The reduction in charge density by the ions was then taken as the difference in charge density between that of a suspension containing either uranyl nitrate or thorium nitrate at a given concentration and that of a control suspension under identical conditions except for the absence of the cation under consideration. The reductions in charge density of the various bacterial derivatives and cardiolipin are plotted against log (normality) of thorium nitrate and uranyl nitrate respectively in Figs. 2 and 3, together with some comparable data of Davies, Haydon and Rideal[7] on *Bacillus coli* and uranyl ions. The curves show that similar reductions of surface charge density are obtained with bacteria, protoplast membranes and defatted protoplast membranes. With the membrane lipid and cardiolipin however, the effects are greater, especially those with uranyl ions. The reversal of charge concentrations of both ions are similar for each of the five types of suspension examined and these too are indicated in Figs. 2 and 3.

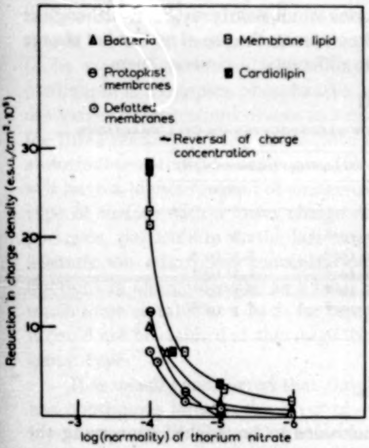

Fig. 2. Reduction in charge density of suspensions of cardiolipin and bacterial derivatives by thorium nitrate.

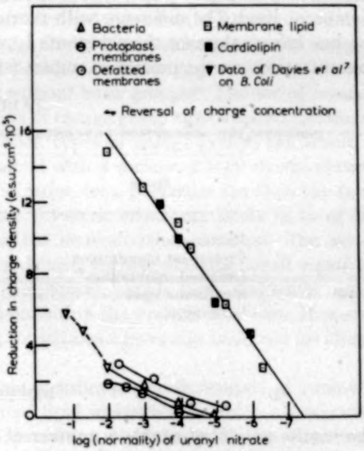

Fig. 3. Reduction in charge density of suspensions of cardiolipin and bacterial derivatives by uranyl nitrate.

In choosing, for purposes of comparison of the systems, any given value of the reduction in charge density, the relative concentration of the ions can vary with the value of charge density reduction selected. This is particularly so with thorium nitrate. For reasons discussed later, it is preferable to use reductions in surface charge density which are small. Further, in order to allow for valency effects and to compare concentrations at equal numbers of ions adsorbed, the comparisons have been made at reductions in charge density which are proportional to the valency of the ion under consideration. A charge density reduction of $1 \cdot 10^3$ e.s.u./cm^2 was chosen for uranyl ions and a reduction of $2 \cdot 10^3$ e.s.u./cm^2 for thorium ions. The concentration of ions producing these reductions in surface charge density is defined as the "isosteric ion adsorption concentration".

The isosteric ion adsorption concentrations are given in Table II. These demonstrate that uranyl ions are extremely potent in producing small reductions in charge density of purely phosphate type colloids, such as cardiolipin and in the protoplast membrane lipid. The difference with thorium ions is not nearly so great, although it too has lower values for the phosphate type. By contrast, the use of reversal of charge concentrations on the present examples fails to differentiate between them.

TABLE II

ISOSTERIC ION ADSORPTION CONCENTRATIONS OF ELECTROLYTES ON CELL FRACTIONS

System	*Isoteric ion adsorption concentration (N)*	
	Th^{4+}	UO_2^{2+}
Bacteria	$1.7 \cdot 10^{-5}$	$2.8 \cdot 10^{-4}$
Protoplast membranes	$2.2 \cdot 10^{-5}$	$6.3 \cdot 10^{-4}$
Defatted membranes	$4.0 \cdot 10^{-5}$	$1.0 \cdot 10^{-4}$
Membrane lipid	$2.2 \cdot 10^{-6}$	$8.3 \cdot 10^{-8}$
Cardiolipin	$2.2 \cdot 10^{-6}$	$8.3 \cdot 10^{-8}$

DISCUSSION

The results presented enable a number of conclusions to be reached concerning the membrane architecture of *M. lysodeikticus* and the ionogenic groups present in its surface.

The pK values derived from the pH–mobility relationships of the protoplast membranes before and after lipid extraction agree closely with those quoted for carboxyl and amine groups in a number of amino acids[5]. Alanine, glutamic acid, glycine and lysine are known constituents of the cell walls of *M. lysodeikticus*[6]. These and several other amino acids have been shown[3] to be present also in the non-lipid fraction of the protoplast membrane. Carboxyl groups with similar pK values may originate also in polysaccharide components[7]. Such components have also been demonstrated in *M. lysodeikticus* in the bacterial cell walls[6] and the defatted protoplast membranes[3]. Precise comparisons with known compounds are not possible because it is not known how the molecular components are linked in the macrostructure of the cell derivatives. However, it appears likely that the electrophoretic behaviour of these biological fractions is controlled by the ionization of carboxyl and amino groups in the surface. Although the membrane lipid has a lower pK than cardiolipin, the acidity of the phosphate group is known to vary with the degree of esterification. The membrane lipid has been shown[3] to contain at least three fractions, the major component of which reacts as a phosphatidic acid. Thus the character of the mobility–pH curve of the membrane lipid is attributed to the presence of phosphate groups. The permanent effects of extremes of pH on the cell membranes of *M. lysodeikticus*[8] appear to occur at a deeper level not contributing to the electrokinetic properties of bacteria or protoplast membranes, since these, held at either pH 2.3 or 10.0, resumed the usual mobility of controls on being returned to neutrality. Similar results have been reported with *Aerobacter aerogenes*[9].

In previous work to elucidate the nature of unknown charge groups by the use of ionic reversal of charge spectra[1], the phosphate colloid models were all phosphatides *e.g.*, lecithin, containing basic groups. For these the reversal of charge concentrations of both uranyl and thorium ions are low (*ca.* $10^{-4}N$). For carboxyl colloid models, however, the reversal of charge concentration of uranyl ions is much higher (approx. 10^{-1} to $10^{-2}N$). The cardiolipin investigated here had a reversal of charge concentration of $10^{-4}N$ for thorium ions and 10^{-2} N for uranyl ions, values previously thought to be more characteristic of carboxyl or possibly sulphate type colloids, whereas cardiolipin is a complex phosphatidic acid without basic groups[10]. The use of reversal of charge concentrations of ions as a criterion of charge group type appears unreliable for three reasons. (a) the coexistence of other types of charge groups can result in abnormal sensitivity to some ions, (b) a colloid with a surface of high charge density will have a higher reversal of charge concentration for a particular ion than the same type of surface with a lower charge density, (c) steric effects are likely to be of importance, particularly in the last stages of the neutralization of charge. The use of isosteric ion adsorption concentrations as defined earlier, utilizing small equal reductions in charge density as a basis for comparison, avoids these difficulties and is much more suitable as a basis for comparison within the systems used here. However, it could not be claimed at this stage that it constitutes a generally valid test for charge group type.

It is usually considered that the plasma membrane of cells consists of a more or less continuous bimolecular layer of oriented lipid molecules on which are adsorbed layers of protein[11]. The evidence for this is almost entirely indirect. The present results provide some experimental support for the general picture as it applies to the bacterial protoplast membrane.

The electrophoretic properties of protoplast membranes are very similar to those of the defatted membranes. This is evident from the pH-mobility curves and the effects of uranyl and thorium ions summarized in Table II. Such minor differences as do exist probably arise from very slight contamination of the defatted membranes with traces of lipid. On the other hand, these properties are entirely dissimilar from those of the membrane lipid. Hence there is strong evidence that the surface of the protoplast membrane does not contain lipid but is similar in nature to the protein–carbohydrate fraction of the membrane. The similar electrophoretic behaviour of protoplasts and protoplast membranes enables these conclusions to be transposed directly in terms of the structure of protoplasts. This study of the electrophoretic properties of the bacterial derivatives thus indicates that the outer layer of the *M. lysodeikticus* protoplast membrane is composed of protein, possibly together with polysaccharide, and that the lipid component must occupy an internal position deeper within the membrane.

ACKNOWLEDGEMENTS

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ÉTUDE DU "RÉTROCONTROLE" DE SYNTHÈSES D'ENZYMES PAR DES ACIDES AMINÉS AU COURS DE LA CROISSANCE DE *PROTEUS MORGANII**

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SUMMARY

Feedback control on synthesis of enzymes by amino acids during growth of P. morganii

The kinetics of growth of *Proteus morganii* shows evidence for a feedback control on the synthesis of enzymes by cysteine and methionine.

These repressions are expressed by lags in the growth and by "diauxies" and "triauxies".

The pool of repressors interferes with the expression of the repression.

INTRODUCTION

Lors de son étude sur la croissance bactérienne, Monod[3] a montré que lorsqu'on fait croître des bactéries en présence de deux sucres dont l'un (a) est métabolisé par des enzymes constitutifs et l'autre (b) par des enzymes adaptatifs, les organismes commencent par utiliser (a) à l'exclusion de (b). Ce phénomène donne lieu à une "diauxie" (croissance double). MONOD concluait: "La diauxie est l'expression d'une variation du pouvoir enzymatique, . . . cette variation est liée à la nature adaptative de certains enzymes et au pouvoir que semblent posséder certains sucres d'inhiber ces enzymes ou plus exactement d'inhiber leur adaptation". La nature de cette inhibition est restée obscure.

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* Une partie de ce travail a fait l'objet de notes préliminaires[1,2].

De nombreux auteurs ont montré, d'autre part, que les bactéries utilisent de préférence un métabolite préformé, plutôt que de le synthétiser *de novo*. Ce "choix" résulte également d'une inhibition dont la nature, dans ce cas, a pu être précisée: il s'agit, cette fois, d'inhibition d'une chaîne métabolique par le produit final résultant de son activité. Ce phénomène a reçu le nom de "feedback control", terme que nous nous proposons de traduire par "rétrocontrôle".

De nombreux cas de rétrocontrôle ont été découverts au cours de ces dernières années: citons notamment les rétrocontrôles par le tryptophane[4], la méthionine[5,6], la valine[7], l'isoleucine[8], l'arginine[9,10], les pyrimidines[11] et les purines[12,13].

De l'ensemble de ces travaux, il ressort que le rétrocontrôle peut s'exercer par deux mécanismes, agissant souvent simultanément: le premier consiste en l'inhibition d'activités enzymatiques le long d'une chaîne métabolique par son produit final, le second en l'inhibition par ce produit de la formation d'enzymes intervenant dans cette chaîne. On réserve à ce second mécanisme le terme de répression[10].

On n'a cependant jamais mis en évidence de diauxie conséquente à un rétrocontrôle. Le présent travail montre dans quelles conditions le rétrocontrôle par la cystéine et la méthionine se manifeste par des latences, des di- et des triauxies.

MATÉRIEL ET MÉTHODES

Microorganisme

Proteus morganii souche P_{18}, provenant du laboratoire du Prof. TULASNE à Strasbourg.

Croissances

Les croissances se font à 36° avec aération par agitation et dans des fioles surmontées d'une cellule cylindrique[14]. On utilise, suivant les cas, des fioles d'une capacité de 125 ml contenant 40 ml de culture, ou des fioles d'une capacité de 1 l contenant 250 ml de culture. Les croissances sont suivies par mesures de D.O. au spectrophotomètre Beckman B à 660 mμ.

Milieux de culture

(a) Milieu d'entretien: "Nutrient Agar" Difco, en tubes inclinés. (b) Milieu minimum (min.): est constitué de glycérol 0.1 M; $(NH_4)_2SO_4$ 0.02 M; tampon aux phosphates (KH_2PO_4–Na_2HPO_4) 0.02 M; NaCl 0.01 M; K_2SO_4 0.01 M; $MgSO_4$ 0.001 M; citrate de fer 0.00004 M; acide nicotinique 10^{-4} M. Le pH final est de 7.3. (c) Milieux complexes: Bouillon: "Nutrient Broth" Difco. 8 g/l sont dissous soit dans du milieu minimum, soit dans du tampon aux phosphates 0.02 M, pH 7.3.

Hydrolysat de caséine: "Casamino Acids – vitamin free" Difco. 8 g/l sont dissous soit dans du milieu minimum, soit dans du tampon aux phosphates 0.02 M, pH 7.3.

Nous avons vérifié que la présence de milieu minimum dans des précultures en bouillon ou en hydrolysat de caséine n'influence pas le comportement ultérieur des bactéries.

Milieu min. acides aminés: Milieu minimum additionné d'un mélange des 19 acides aminés purs constituant approximativement la caséine, soit, par litre de milieu 0.35 g L-arginine, 0.28 g L-histidine, 0.71 g L-lysine, 1.92 g acide L-glutamique (neutralisé), 0.58 g acide L-aspartique (neutralisé), 0.03 g glycine, 0.28 g L-alanine, 0.52 g L-valine, 0.91 g L-leucine, 0.45 g L-isoleucine, 0.92 g L-proline, 0.56 g L-phénylalanine, 0.102 g DL-sérine, 0.78 g DL-thréonine, 0.45 g L-tyrosine, 0.21 g L-hydroxyproline, 0.23 g L-tryptophane, 0.03 g L-cystéine, 0.58 g DL-méthionine.

Produits

Tous les sels utilisés sont de qualité "pro analysi". La DL-allocystathionine et la DL-homocystéine sont des produits de la "California Foundation for Biochemical Research". La DL-méthionine est un produit de la "Nutritional Biochemicals Co.". Tous les autres acides aminés sont des produits "F. Hoffmann-La Roche und Co.". L'acide L-glutamique Hoffmann-La Roche. contient des traces d'autres acides aminés et, notamment, de la méthionine. Il a été purifié par 4 recristallisations dans l'eau.

Récolte

Les bactéries sont récoltées à la surface d'un tube de gélose incliné âgé de 15 h environ, et mises en suspension dans le milieu de préculture de façon à réaliser une D.O. de 0.1 environ. Quand la préculture a atteint la D.O. de 0.350, les bactéries sont récoltées stérilement par centrifugation, remises en suspension dans le milieu minimum et centrifugées une seconde fois. Le culot de bactéries lavées est alors remis dans quelques millilitres de milieu minimum. Cette suspension dense est utilisée à l'ensemencement des milieux à expérimenter. La valeur de l'inoculum n'influence pas le sens des résultats. Toutefois, on s'efforce d'ensemencer toutes les fioles d'une même expérience à la même D.O. de départ, généralement 0.1 environ.

RÉSULTATS EXPÉRIMENTAUX

Influence du milieu de préculture sur la latence en milieu minimum

Si on fait croître *Proteus morganii* en milieu minimum et, après centrifugation et lavage, on le transfert dans le même milieu, la croissance reprend immédiatement. Par contre, si on transfert de la même façon en milieu minimum des bactéries provenant d'une préculture en présence de bouillon nutritif, la croissance ne reprend qu'après une longue latence. Celle-ci varie d'une expérience à l'autre, de 24 à 60 h.

Cette latence est due aux acides aminés contenus dans le bouillon de préculture car on l'observe également après croissance en présence d'hydrolysat de caséine ou du mélange des 19 acides aminés constituant la caséine (milieu min. acides aminés) (Fig. 1).

Nous avons vérifié cette croissance tardive n'est pas due à une sélection de mutants.

Cette latence suggère que les organismes sont devenus incapables de synthétiser une ou plusieurs substances essentielles à leur croissance. Afin de préciser leur déficience, nous avons testé l'effet de nombreux composés sur cette latence.

Effet d'acides aminés soufrés sur la latence

Les acides aminés soufrés L-cystéine et DL-méthionine, et leurs dérivés, la DL-homocystéine et la DL-allocystathionine, sont les plus actifs à réduire la latence en milieu minimum. Leur addition à faible concentration réduit cette latence à 2 h environ (Fig. 2). L'homosérine et la sérine ne raccourcissent pas la latence.

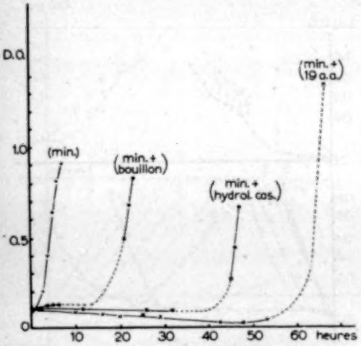

Fig. 1. Croissances en milieu minimum de *Proteus morganii* ayant crû au préalable dans le milieu indiqué entre parenthèses.

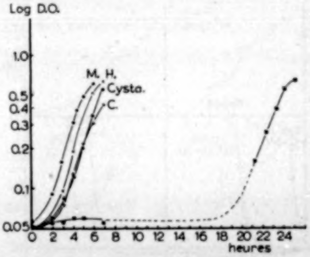

Fig. 2. Croissances en milieu minimum de *Proteus morganii* ayant crû au préalable en bouillon tamponné. ■—■ min.; ●—● min. + DL-méthionine 0.0002 *M*; ×—× min. + DL-homocystéine 0.0002 *M*. +—+ min. + L-cystéine 0.0001 *M*; ○—○ min. + DL-allocystathionine 0.0002 *M*.

Effet sur la latence de la suppression de la cystéine et de la méthionine lors de la préculture

Les résultats précédents suggèrent que la croissance en milieu complexe rend *P. morganii* incapable de synthétiser la cystéine et la méthionine. On peut penser que la perte de cette capacité biosynthétique est due à la présence, dans la préculture, de cystéine et méthionine qui réprimeraient la synthèse d'enzymes intervenant dans leur propre voie biosynthétique. L'expérience représentée par la Fig. 3 est une première preuve qu'il en est bien ainsi.

Un témoin, ayant crû en présence de 19 acides aminés (milieu min. acides aminés), présente la longue latence habituelle. La suppression du milieu de préculture soit de la cystéine, soit de la méthionine, réduit considérablement cette latence. La réduction de la latence est renforcée si on supprime simultanément la cystéine et la méthionine. Ceci suggère que, quoique faisant partie de la même chaîne métabolique, chacun y exerce une répression sur la synthèse d'enzymes différents. Il semble, d'autre part, que la cystéine soit responsable de la majeure partie de la latence.

Diauxie en présence de cystéine

Comme nous l'avons montré ci-dessus (Fig. 2), des bactéries réprimées par croissance en milieu complexe, puis transférées en milieu minimum additionné de L-cystéine, y croissent après une courte latence (2 h). Cependant, en présence d'une dose de L-cystéine suffisamment faible, $6.2 \cdot 10^{-5}$ *M* par exemple, nous observons un phénomène de diauxie (croissance double)[3]. La croissance se déroule en deux phases exponentielles séparées par une phase de croissance ralentie.

Afin d'interpréter ce phénomène, nous avons déterminé, au cours d'une telle diauxie, l'état de répression des bactéries en mesurant la latence lors de leur transfert en milieu minimum. Les résultats sont représentés dans la Fig. 4.

La phase de croissance ralentie apparaît comme une manifestation de l'effet répresseur de la cystéine. En effet, les bactéries récoltées au point 3, c'est-à-dire peu avant le ralentissement, transférées en milieu minimum, n'y croissent qu'après une latence de 24 h environ (courbe inférieure 3). Elles sont donc fortement réprimées.

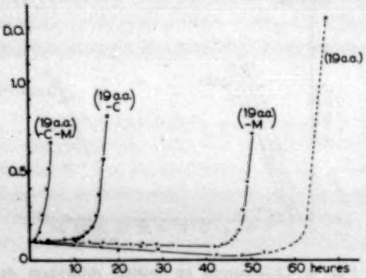

Fig. 3. Croissances en milieu minimum de *Proteus morganii* ayant crû au préalable dans les milieux suivants: ×—× min. acides aminés; +—+ min. acides aminés sans méthionine; O—O min. acides aminés sans cystéine; ●—● min. acides aminés sans méthionine, ni cystéine.

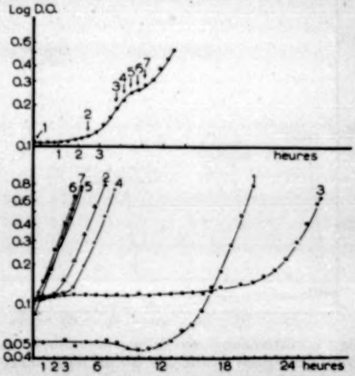

Fig. 4. Courbe supérieure: diauxie en présence de L-cystéine $6.2 \cdot 10^{-5}$ *M*. Préculture en milieu minimum additionné de bouillon. Courbes inférieures: croissances en milieu minimum de *Proteus morganii* récolté en différents points de la diauxie.

Au point 4, la répression est déjà beaucoup plus faible et a disparu aux points 5, 6 et 7. Le ralentissement de la croissance correspond donc à l'épuisement de la cystéine du milieu, accompagné de la levée de la répression qu'elle exerçait.

L'état des bactéries, au point 2, est remarquable et fait ressortir un phénomène nouveau. Les bactéries sont fortement réprimées aux points 1 et 3. Le point 2 se situe donc en pleine période de répression et, cependant, ces bactéries se comportent en milieu minimum comme très faiblement réprimées (latence de 2 h). Cette anomalie apparente nous a suggéré l'explication d'un phénomène constaté antérieurement lors de l'étude du degré de répression en fonction de la concentration en cystéine du milieu complexe. Il était apparu que, si on augmente la dose de cystéine du milieu complexe, à partir d'une certaine concentration la répression, loin d'être plus forte, semble partiellement levée.

On peut penser que, dans un milieu déséquilibré par la présence d'un excès de cystéine (comme c'est le cas au point 2 où la cystéine est le seul acide aminé présent), les bactéries accumulent une réserve intérieure de cystéine libre (pool), qu'elles emportent lors de leur transfert en milieu minimum, où le départ de la croissance s'en trouve facilité.

Diauxie en présence de méthionine

La répression par la méthionine se manifeste également, en présence de DL-méthionine $3.1 \cdot 10^{-5}$ *M*, par une diauxie. L'état de répression donne également lieu, lors des transferts en milieu minimum, à des latences (points 4 et 5 de la Fig. 5).

L'accumulation d'un pool de méthionine masque également le phénomène aux points 1, 2 et 3. Cette accumulation semble particulièrement rapide: au point 1, les bactéries provenant du milieu complexe n'ont séjourné que pendant quelques minutes en milieu minimum additionné de méthionine seule. Elles croissent en milieu minimum après une latence de 10 h environ, nettement inférieure à celle que l'on aurait observée par transfert direct des bactéries de milieu complexe en milieu minimum (Fig. 1).

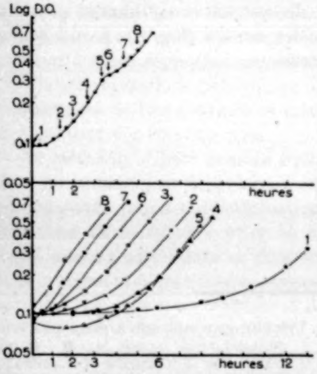

Fig. 5. Courbe supérieure: diauxie en présence de DL-méthionine $3.1 \cdot 10^{-5}$ *M*. Préculture en bouillon tamponné. Courbes inférieures: croissances en milieu minimum de *Proteus morganii* récolté en différents points de la diauxie.

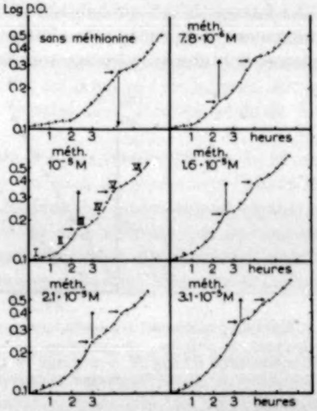

Fig. 6. Triauxies en milieu minimum additionné de L-cystéine $6.2 \cdot 10^{-5}$ *M* et de DL-méthionine aux concentrations indiquées. Préculture en hydrolysat de caséine tamponné.

Triauxie en présence de cystéine et méthionine simultanément

La Fig. 6 met en évidence une triauxie correspondant aux deux répressions distinctes exercées par la cystéine et la méthionine.

La croissance se déroule en trois phases exponentielles successives (II, IV et VI), précédées chacune d'une phase de croissance ralentie (I, III et V).

En faisant varier la dose de méthionine et en maintenant constante la dose de cystéine, la valeur de la phase de croissance II varie de façon approximativement proportionnelle à la dose de méthionine, tandis que la croissance IV reste constante. Si l'on surmonte la fin de la phase II de la valeur (en coordonnées logarithmiques) de l'accroissement de D.O. obtenu en présence de cystéine seule, on atteint, dans chaque cas, une valeur très proche de la phase V.

On peut donc conclure que la croissance II correspond à l'utilisation de la méthionine du milieu jusqu'à épuisement, atteint au début de la phase III. Cet épuisement est accompagné de l'apparition d'enzymes de biosynthèse de la méthionine à partir de cystéine. *Proteus morganii* utilise alors la cystéine du milieu, ce qui correspond à la croissance IV, jusqu'à épuisement de la cystéine, en V, accompagné de l'apparition d'enzymes de biosynthèse de cystéine. A la fin de la phase de ralentissement V, les bactéries sont devenues capables de synthétiser la cystéine (à partir de glycérol, d'ammonium et de sulfate) et la méthionine (à partir de cystéine), donc de croître en milieu minimum, ce qui correspond à la phase VI.

Nature de la latence résiduelle

La latence de 2 h qui subsiste en présence de cystéine et méthionine peut être, pratiquement, supprimée par addition de 7 acides aminés (Fig. 7), parmi lesquels l'arginine, la lysine et la leucine sont les plus actifs.

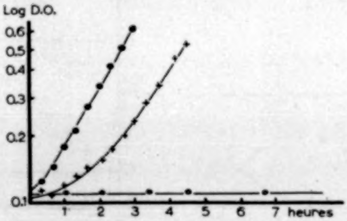

Fig. 7. Effet de 7 acides aminés sur la latence résiduelle. Préculture en milieu minimum additionné d'hydrolysat de caséine. ○—○ min.; ×—× min. + DL-méthionine 0.0005 *M*; ●—● min. + DL-méthionine 0.0005 *M* + L-lysine + L-arginine + L-histidine + L-leucine + L-isoleucine + glycine + L-tryptophane 0.0001 *M* chacun.

Cette latence résiduelle correspond aussi à des répressions exercées par ces acides aminés. Ces répressions sont, toutefois, levées beaucoup plus rapidement (2 h) que celles exercées par la méthionine et la cystéine (40 h en moyenne).

DISCUSSION

L'étude de la cinétique de croissance de *Proteus morganii* dans les conditions décrites ci-dessus constitue une méthode simple, et peut-être générale, de mise en évidence de phénomènes de rétrocontrôle par le mécanisme de répression de synthèses d'enzymes. Cette méthode, toutefois, ne permet pas de déceler les rétrocontrôles par inhibition d'activités enzymatiques.

Les répressions de synthèse d'enzymes s'expriment dans ce travail par deux phénomènes: d'une part, par les diauxies observées en présence de répresseur et, d'autre part, par les latences qui s'écoulent lors du transfert en milieu minimum de bactéries réprimées. Dans ces deux cas, l'éloignement de l'acide aminé—soit par épuisement, soit par transfert—lève l'inhibition éventuelle de l'activité enzymatique, et le ralentissement de croissance ou la latence ne peuvent être dûs qu'à l'absence des enzymes spécifiques de synthèse des acides aminés en cause. La latence en milieu minimum constitue une mesure indirecte du contenu enzymatique des cellules.

Il semble qu'on puisse conclure également que la levée de la repression s'accomplit par une synthèse *préférentielle* des enzymes réprimés, comme UMBARGER ET BROWN l'ont montré[15]. En effet, la répression est totalement levée en 40 min, alors que l'accroissement de la masse bactérienne n'est que de 30 % (comparer les points 3 et 5 de la Fig. 4). La synthèse des enzymes préalablement réprimés s'accomplit en utilisant la dernière trace d'acide aminé répresseur, dès que celui-ci a atteint une concentration non répressive. Ceci ressort particulièrement de la différence de temps nécessaire à cette synthèse d'enzymes selon qu'elle s'effectue à la suite de l'épuisement du répresseur (diauxie) ou à la suite d'un transfert en milieu minimum des bactéries réprimées et lavées. Ce transfert élimine, en effet, l'acide aminé répresseur indispensable à la restauration enzymatique. La dégradation que subissent les protéines pendant la latence[16] constitue alors la seule source des acides aminés dont la synthèse est réprimée. La nécessité du represseur pour effectuer la restauration enzymatique est propre à toute repression au niveau de la biosynthèse d'un acide aminé et rend la levée de ces répressions particulièrement difficile; les latences très longues, souvent accompagnées de lyse partielle et même, dans certains cas, l'absence totale de croissance, en sont des conséquences.

La méthode utilisée dans ce travail, fondée uniquement sur l'étude de la croissance, ne permet pas de préciser entièrement les sites de répression. Celle-ci doit être complétée par des déterminations quantitatives des enzymes participant à la biosynthèse de la cystéine et de la méthionine, et doit attendre que de nouveaux progrès soient réalisés dans ce domaine. On peut cependant, dès à présent, conclure que la cystéine et la méthionine agissent au moins en deux sites différents de la chaîne biosynthétique, sinon on n'observerait pas de triauxie.

Le(s) site(s) de répression de la méthionine se situent-ils uniquement entre la cystéine et la méthionine ou intéressent-ils des stades de la synthèse de la cystéine? Nous avons examiné l'état de *Proteus morganii* réprimé par croissance en présence de méthionine seule: lors du transfert en milieu minimum, la latence, qui est évidemment supprimée par la méthionine, subsiste en partie (90 min) en présence de cystéine. On peut donc localiser une répression entre la cystéine et la méthionine. A première vue, ceci implique que la méthionine réprime la synthèse de la cystéine, mais ceci peut être dû à la présence de cystéine intracellulaire dérivée de la méthionine par une voie différente de l'inverse de la voie réprimée.

Le fait que l'on retrouve au cours des triauxies une quantité constante de cystéine après l'utilisation de quantités variables de méthionine suggère que la méthionine inhibe l'utilisation de la cystéine. Ce fait inattendu ne pourra être compris entièrement que par l'utilisation d'une méthode moins indirecte que celle de la croissance.

Soulignons enfin l'importance de l'accumulation d'un pool de répresseur dans la mise en évidence de la répression. Le comportement de bactéries récoltées au cours d'une croissance en présence d'un excès de répresseur est la résultante de deux phénomènes qui se neutralisent: répression et accumulation de répresseur. L'état réprimé ne se manifestera donc que chez les bacteries récoltées au moment où la concentration en répresseur est telle que le pool soit négligeable et où la répression n'est pas encore levée. Cet état est très transitoire car, dès que cette faible concentration en répresseur est atteinte, la restuaration enzymatique s'accomplit rapidement. Ces phénomènes rendent compte d'échecs rencontrés lors d'essais de mise en évidence de répression après croissance en présence d'un excès de répresseur. On entend par excès de répresseur, une concentration anormalement élevée par rapport aux autres acides aminés du pool.

En plus de l'intérêt immédiat du rétrocontrôle pour l'économie de la cellule, cette notion permet d'interpréter des latences et des irrégularités fréquemment rencontrées au cours des cultures bactériennes.

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RÉSUMÉ

L'étude de la cinétique de croissance de *Proteus morganii* a permis de mettre en évidence des phénomènes de répression de synthèse d'enzymes par la cystéine et la méthionine.

Ces répressions se manifestent par des latences, ainsi que par des diauxies et des triauxies.

Les réserves intracellulaires de ces répresseurs (pool) interfèrent avec l'expression de la répression.

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Biochim. Biophys. Acta, 38 (1960) 136–144

Short Communications

Studies on the N-terminal sequence of tobacco-mosaic-virus protein

In a recent paper from this laboratory, NARITA reported the separation of approximately one mole of N-acetylseryltyrosine from the chymotryptic and peptic digests of the protein of tobacco mosaic virus[1]. Even though his work suggested that the acetyl peptide was located in the N-terminal position of TMV-protein, there remained the possibility that the acetylpeptide was derived from a branch chain linked to the ε-amino group of one of the lysine residues of TMV-protein[1,2]. Definite evidence for the location of N-acetylseryltyrosine at the N-terminus of the protein is reported in this paper.

The following three products were investigated: a chymotryptic digest of TMV-protein and synthetic N-acetylseryltyrosine, both prepared according to NARITA[1]; and a chymotryptic digest of the insoluble peptide (I-peptide) obtained as a precipitate at pH 4.5–4.7 after digestion of TMV-protein with trypsin. From a study of the I-peptide[3] it was known that it consists of about 40 amino acid residues and that it does not contain a lysine residue nor any amino terminal residue which is reactive to dinitrofluorobenzene. This peptide was digested to a point where 3.7 moles of amino residues were liberated.

The three materials were chromatographed under almost identical conditions on a column (0.9 × 20 cm) of Dowex 50 X 2 in hydrogen form. The absorption of the effluent was measured at 280 mμ, and those tubes which showed absorption were again measured from 300 to 250 mμ using the Cary recording spectrophotometer. The fractions which showed the typical absorption spectrum of tyrosine were pooled. An aliquot of each fraction was made 0.1 *N* in NaOH and the absorption was measured. The absorption value obtained and the volume of each fraction gave the recovery of acetylseryltyrosine of each sample based on the molecular extinction coefficient of tyrosine. The results are shown in Table I.

Lastly, to confirm that the absorption values observed with the digests were due to acetylseryltyrosine, the two peptide fractions obtained from the chymotryptic digests of I-peptide and of TMV-protein were hydrolyzed by acid and analyzed for

TABLE I

RECOVERY OF ACETYL PEPTIDE FROM I-PEPTIDE, TMV-PROTEIN AND SYNTHETIC PEPTIDE

	Amount used (μmoles)	*Amount recovered (μmoles)**	*Recovery (%)*
I-peptide	4.95	3.93	79.4
TMV-protein	4.47	3.47	77.6
Acetylseryltyrosine	3.96	3.21	81.1

* These values are calculated using a molecular extinction coefficient of $1.97 \cdot 10^{-3}$ for tyrosine in 0.1 *N* NaOH at 284 mμ.

Abbreviation: TMV, tobacco mosaic virus.

amino acids by the dinitrophenylation method. In both cases, it was found that tyrosine and serine were present in a one to one molar ratio.

As these experiments indicated, about 0.8 mole of the acetyl peptide/mole was obtained from the chymotryptic digests of I-peptide and TMV-protein. The synthetic acetyl peptide was recovered in a similar yield. The loss of 20 % might be caused by an acetyl shift from N to O under the conditions of the chromatography used. In conclusion, the good agreement in recovery in all chromatographic treatments suggests that the I-peptide actually contains one mole of N-acetylseryltyrosine.

The fact that the I-peptide does not contain lysine indicates that the acetyl peptide is located in the N-terminal position of the I-peptide and the possibility of branching from the ε-amino group of lysine must be excluded. Thus it may be concluded that N-acetylseryltyrosine occupies the N-terminal position of TMV-protein and of the I-peptide located at the N-terminus of the TMV-peptide chain.

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The stabilization of unstable amino acids in polypeptides by desulphurization and hydrogenation using tritium

The instability of several amino acids during the cleavage of peptide chains presents one of the main problems encountered in the investigation of protein structures. Major difficulties in this field are caused by tryptophan which is destroyed by the process of hydrolysis by acid and during oxidation by performic acid of sulphur-containing amino acids. Further obstacles arise from the instability of tyrosine, cysteine and methionine.

Making use of our previous experience[1] we attempted to overcome these procedural difficulties by transforming tryptophan into the stable octahydrotryptophan derivative by hydrogenation. The application of this procedure to protein hydrolysates, however, requires desulphurization to be performed as a first step, since the hydrogenation of tryptophan is impeded by sulphur-containing amino acids. On desulphurization, cysteine is converted into alanine, and methionine into α-aminobutyric acid. Using tritium, alanine arising from cysteine may be labelled and thus distinguished from the original alanine residues of the protein molecule. The hydrogenation of tryptophan is paralleled by the reduction of tyrosine and phenylalanine.

A chromatographic procedure involving the passage of solutions of sulphur-containing amino acids or the corresponding peptides through a column of Raney nickel[2] operated with cooling was used for desulphurization. When using 400 mg of Raney nickel (column dimensions 10 × 3 mm) quantitative desulphurization of 5 mg cysteine and 0.7 mg methionine was obtained. Obviously, the total desulphurizing capacity of the column is much higher, but the filtrate then contains both the desulphurized and the original material. Under the same conditions, glutathione was transformed quantitatively to pure γ-glutamylalanylglycine. Using this procedure the losses in aromatic peptides due to adsorption are insignificant.

The hydrogenation of aromatic amino acids depends both on pH and on the catalyst used. In the acidic range, and in the presence of palladium or platinum, tryptophan and phenylalanine are readily converted into the basic octahydrotryptophan and cyclohexylalanine, respectively. In the case of tyrosine, the formation of a mixture of hexahydrotyrosine and cyclohexylalanine in the slightly acidic or neutral range was observed, in accordance with the findings of WASER AND BRAUCHLI[4]. The ratio of these compounds cannot be influenced by a change of catalyst, *e.g.* palladium for platinum. From our observations[3], hexahydrotyrosine is unstable during acid hydrolysis, being decomposed mainly into cyclohexenylalanine. On hydrogenation in the acid (0.08 N to 0.1 N HCl) and using the platinum catalyst, tyrosine is transformed only into cyclohexylalanine, which is quite stable under the conditions of hydrolysis.

The procedures described have been carried out also using tritium. The technical process of the desulphurization reaction is very simple, since it consists in passing a solution of the substance through a column of Raney nickel, previously equilibrated on standing with ^3H_2O. In a typical experiment cysteine was converted to [^{3}H]alanine with a stable specific activity of 4 mC/mmole. The hydrogenation reactions were carried out in a closed system where gaseous tritium was developed by the decomposition of ^3H_2O by metallic lithium. The technical process is more complicated in comparison with that used for desulphurization, since an excess of isotope is required. In a typical experiment a chromatographically homogeneous sample of octahydrotryptophan possessing a stable specific activity of 60 mC/mmole was obtained from tryptophan. In all cases the isotope was removed from ionizable groups by repeated lyophilization.

The application of the procedures described to a peptic hydrolysate of chymotrypsinogen resulted in the conversion of cysteine, methionine, and the aromatic amino acids residues into [^{3}H]alanine, [^{3}H]α-amino-butyric acid, and [^{3}H]octahydrotryptophan or [^{3}H]cyclohexylalanine, respectively. All these compounds are stable during the subsequent treatment. It is in our opinion that the procedure described may be of general value in the elucidation of protein structures.

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Paper-chromatographic differentiation between α-monoamino acids and other ninhydrin-positive substances

In the course of studies in this laboratory on certain derivatives of α,β-diaminopropionic acid[1] a need arose for a paper-chromatographic method for distinguishing between α-monoamino acids and other ninhydrin-positive compounds including amino derivatives of the latter. Previously published methods[2–4] utilizing complex formation between all α-amino acids and Cu^{++} did not prove satisfactory for the present purpose. Hence, an alternative procedure has been developed, based on specific masking of the α-amino acid grouping with Cu^{++} to such an extent as to make it virtually insusceptible to subsequent ninhydrin treatment.

The dried chromatograms, run in standard solvent systems, are dipped through a methanolic solution containing 0.25 % $Cu(NO_3)_2 \cdot 3H_2O$ and 2 % anhydrous CH_3COONa, and dried for 2 min at 70°. Subsequent dipping of the chromatograms

TABLE I

COLOUR REACTIONS AND INTENSITIES OF VARIOUS AMINO-SUBSTITUTED COMPOUNDS AFTER NINHYDRIN TREATMENT WITH AND WITHOUT PRETREATMENT WITH COPPER SALT

Compound	*Development with ninhydrin*				*Development with Cu^{++}-reagent followed by ninhydrin*			
	Immediately after treatment		*20 h after treatment*		*Immediately after treatment*		*20 h after treatment*	
	*Colour**	*Intensity***	*Colour**	*Intensity***	*Colour**	*Intensity***	*Colour**	*Intensity***
1. L-Alanine	V	++	V	+++	V	((+))	V	(+)
2. L-α-Aminobutyric acid	V	++	V	+++	V	((+))	V	(+)
3. L-Valine	V	++	V	+++	V	((+))	V	(+)
4. L-Leucine	V	++	V	+++	V	((+))	V	(+)
5. L-Isoleucine	V	++	V	+++	V	((+))	V	(+)
6. L-Serine	V	++	V	+++	V	((+))	V	(+)
7. L-Threonine	V	++	V	+++	V	((+))	V	(+)
8. L-Methionine	V	++	V	+++	V	((+))	V	(+)
9. L-Aspartic acid	V	+	V	++			Y-Bn	(+)
10. L-Glutamic acid	V	++	V	++	V	((+))	V	(+)
11. DL-α-Phenylglycine	Y	+	V	++			Y-Bn	(+)
12. L-Phenylalanine	V	++	V	++			V	(+)
13. L-Tyrosine	V	((+))***	V	+			V	(+)
14. L-Histidine	V	+	V	++	Y-Gr	((+))	Y-Bn	(+)
15. L-Tryptophane	V	+	V	++	V	((+))	V	(+)
16. L-α-Amino-γ-guanidinobutyric acid	V	++	V	++	V	((+))	V	(+)
17. L-Arginine	V	++	V	++	V	((+))	V	(+)
18. L-α-Amino-ε-guanidinocaproic acid	V	++	V	++	V	((+))	V	(+)
19. L-α-Amino-β-ureidopropionic acid§ (Albizziine)	V	++	V	++	V	((+))	V	(+)
20. L-α-Amino-γ-ureidobutyric acid	V	++	V	++	V	((+))	V	(+)
21. L-Citrulline	V	++	V	++	V	((+))	V	(+)
22. L-α-Amino-ε-ureidocaproic acid	V	++	V	++	V	((+))	V	(+)

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[*] The following abbreviations have been used to indicate the colours: V, violet; Y, yellow; R, red; O, orange; Bn, brown; Gr, grey.

[**] Intensities are specified as follows: +++, very strong; ++, strong; +, medium; (+), faint and ((+)), very faint.

[***] The weakness is due to the limited solubility of tyrosine in water.

[§] New compounds (*cf.* ref.[1]).

through an 0.2 % solution of ninhydrin in acetone is followed by air-drying for 1 min and heating at 70° for 40–50 sec.

Whereas α-monoamino acids under these conditions do not at all appear, or at most as very faint violet spots, other amino acids, such as α,β-diaminopropionic acid, β-alanine, γ-aminobutyric acid, ornithine and lysine, rapidly produce intense and characteristic, individual colours. Glycine represents the sole exception to the above rule because this amino acid as well as a number of glycyl derivatives yield persistent yellow or orange spots, which may possibly be of diagnostic value for the identification of glycine and glycyl peptides. On prolonged keeping at room temperature all α-amino acids produce visible violet spots, but of weak intensity compared to those on the chromatograms exclusively treated with ninhydrin.

In Table I are listed the results obtained with 10 μg of each of 50 individual, ninhydrin-positive compounds subsequent to paper chromatography on Whatman paper No. 1 in *n*-butanol–acetic acid–water (12:3:5). Chromatographic results obtained with other solvent systems have been equally consistent and the procedure

TABLE I, *continued*

Compound	*Development with ninhydrin*				*Development with Cu++ reagent followed by ninhydrin*			
	Immediately after treatment		*20 h after treatment*		*Immediately after treatment*		*20 h after treatment*	
	*Colour**	*Intensity***	*Colour**	*Intensity***	*Colour**	*Intensity***	*Colour**	*Intensity***
23. L-Cysteine·HCl	V	+	V	+			Y-Bn	(+)
24. L-Asparagine	V	+	R-Bn	+			Gr-Bn	(+)
25. L-Proline	Y	+	Y	+	Y	((+))	Gr-Bn	(+)
26. β-Alanine	V	((+))	V	+	Bn	++	Bn	++
27. Taurine	V	(+)	V	+	R-Bn	++	R-Bn	++
28. L-α, β-Diaminopropionic acid·HCl	V	++	V	++	Y-Bn	++	Y-Bn	++
29. L-α-Ureido-β-amino-propionic acid§	V	(+)	V	+	Y-Bn	++	Y-Bn	++
30. γ-Aminobutyric acid	V	+	V	++	R	++	R	++
31. L-α, γ-Diaminobutyric acid	V	++	V	++	Y-Bn	++	R-Bn	++
32. L-Ornithine·2HCl	V	++	V	++	R-V	++	R-V	++
33. L-Lysine·HCl	V	++	V	++	R-V	++	R-V	++
34. Glycine	V	++	V	++	O	++	O	++
35. Glycylglycine·HCl	Y-Bn	+	V	++	Y	(+)	Y	+
36. Glycylglycylglycine	Y-Bn	+	V	++	Y	(+)	Y	+
37. Glycyl-L-leucine	R-Bn	+	V	++	Y	(+)	Y	+
38. Glycyl-L-tryptophane			V	+	Y	(+)	Y	+
39. Glycinamide, $0.5H_2SO_4$	Y-Bn	+	V	++	Y	(+)	Y	+
40. Glycine methyl ester,HCl	Y	+	V	++	Y	(+)	Y	+
41. DL-Alanylglycine	V	+	V	++	Y	(+)	R-Bn	+
42. L-α-Glutamyl-L-methionine	V	+	V	++	Y	(+)	V	+
43. L-Tyrosinamide, $0.5H_2SO_4$	V	+	V	++	Y	(+)	Y-Bn	+
44. DL-Alanine methyl ester·HCl	V	+	V	++	Y	(+)	V	+
45. L-Leucine ethyl ester·HCl	V	+	V	+	Y	(+)	V	+
46. DL-Phenylalanine ethyl ester·HCl	V	+	V	++	Y	(+)	Y-Bn	+
47. Ethanolamine	V	+	V	++	V	+	R-V	++
48. 3-Amino-1-propanol	V	+	V	++	V	+	R-V	++
49. Benzylamine	Y	++	V	++	Y	+	R-Bn	++
50. Ethylendiamine	V	++	V	++	R-V	+	R-Bn	++

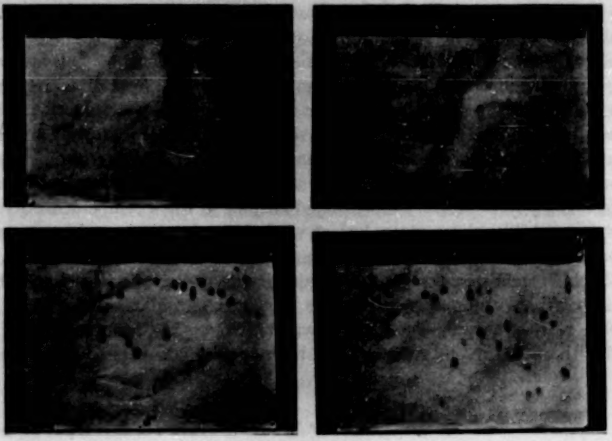

Fig. 1. Photographic reproduction of descending paper chromatograms of the compounds No. 1–50, listed in Table I, on Whatman paper No. 1 in *n*-butanol–acetic acid–water (12 : 3 : 5). Upper row: ninhydrin-treated papers after impregnation with copper salt. Lower row: Same compounds treated with ninhydrin without copper impregnation.

has proved useful also in spot-test technique on filter paper. Photographic reproductions of ninhydrin-treated paper chromatograms of all compounds listed in Table I on copper-impregnated as well as untreated paper are presented in Fig. 1.

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Chloramphenicol and chlortetracycline inhibition of amino acid incorporation into proteins in a cell-free system from *Tetrahymena pyriformis*

Growth of *Tetrahymena pyriformis* "W" in 2.5% proteose peptone (Difco) was considerably delayed or completely arrested by chloramphenicol at the relatively high levels ranging from 25 to 150 μg/ml medium. Similar concentrations of the

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Abbreviations: ATP, GTP, CTP, UTP, the triphosphates of adenosine, guanosine, cytidine and uridine respectively; PEP, phosphoenol pyruvate; RNA-ase, ribonuclease; Tris, tris(hydroxymethyl)aminomethane.

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antibiotic produced a 30 to 60 % inhibition of amino acid incorporation into protein in both intact cells and in crude cell-free homogenates. Thus, the action pattern of chloramphenicol in *T. pyriformis*, notwithstanding the low order of susceptibility of this protozoon, conformed essentially with its mode of action in bacteria as revealed by the study of GALE AND FOLKES[1].

It appeared puzzling, therefore, that the previously described microsomal system for amino acid incorporation into protein, which has been isolated by differential centrifugation from crude homogenates of *T. pyriformis*[2], proved to be refractory to chloramphenicol added in amounts as high as 250 μg/ml reaction mixture. This finding, agreeing with results obtained in similar experiments with microsomal preparations from rat and guinea-pig liver (prepared according to KELLER AND ZAMECNIK[3]) suggested the possibility that some protein-synthesizing system, other than the microsomal system, may be responsible for the inhibitory effect of chloramphenicol observed *in vivo* and in the whole homogenates. In fact, the existence of such a system could be demonstrated in the heavy subcellular fraction left after separation of the microsomal particles. This fraction which was mainly composed of mitochondria will be referred to as the "mitochondrial system" although it is not at all certain that the amino acid incorporation activity adheres to the mitochondrial structure.

The capacity of the mitochondrial system to incorporate amino acids into its proteins was found to be ATP-dependent and was significantly enhanced by addition of a mixture of GTP, CTP and UTP. Its specific activity in terms of amounts of [^{14}C]leucine incorporated per mg protein was 3- to 4-fold lower in the mitochondrial than in the microsomal preparation. The saturating level of [^{14}C]leucine required for optimal incorporation was about $5 \cdot 10^{-4}$ *M* *i.e.* nearly 5 times higher than the corresponding value for the microsomal system. Again in contrast to the microsomes, the presence of cell sap (supernatant resulting from centrifugation of the homogenate at 105,000 × *g* for 1 h) did not appear essential for the activity of the mitochondria, under the conditions employed, since a twice-washed preparation showed only slight stimulation on addition of cell sap. The two distinctive characteristics, however, sharply differentiating the mitochondrial system from its microsomal counterpart were: resistance to treatment with ribonuclease and susceptibility to strong inhibition by chloramphenicol. A comparative summary of the salient features of the two systems is presented in Table I.

Omission of the extraction with hot trichloroacetic acid from the standard procedure used for assaying the incorporation of the radioactive amino acid into protein[2,3] revealed the appearance of labeled non-protein material in the chloramphenicol-containing reaction mixtures. In the control experiments on the other hand, all the radioactivity incorporated appeared to be protein-bound, as evidenced by the practically identical values obtained by either the standard assay or the modified procedure (see Table II). In the light of the latter observation it appears reasonable to assume that chloramphenicol causes the accumulation of an amino acid compound which is precipitable by cold trichloroacetic acid and destroyed by heating in trichloroacetic acid at 90° for 10 min. The labile nature of the amino acid linkage in the above compound was further indicated by the finding that, following brief exposure to 1 *N* NaOH or to neutralized 0.5 *M* NH_2OH, all the radioactivity was split off, as evident from the fact that the labeled amino acid was no more precipitable by cold trichloroacetic acid but remained in the trichloroacetic acid supernatant.

TABLE I

FACTORS INFLUENCING AMINO ACID INCORPORATION INTO PROTEINS OF THE MITOCHONDRIAL AND MICROSOMAL SYSTEM

The complete system contained in 1 ml total volume the following ingredients: 100 µmoles Tris buffer pH 7.4, 120–150 µmoles sucrose, 25 µmoles KCl, 5 µmoles $MgCl_2$, 1 µmole ATP, 5 µmoles PEP, 25 µg crystalline pyruvate kinase, 0.4 µmole L-[1-^{14}C]leucine (about $1.2 \cdot 10^6$ counts/min), cell sap (2–3 mg protein) and either mitochondria twice-washed in 0.25 *M* sucrose (8–10 mg protein) or microsomes (4–6 mg protein) prepared as previously described[2]. Where indicated the following compounds were added: nucleoside triphosphates: GTP, CTP and UTP, 0.25 µmole each; crystalline ribonuclease (Worthington), 50 µg; chloramphenicol, 100 µg; chlortetracycline, 100 µg. Incubation, 30 min at 30°. Assay and procedure as described[2].

Reaction mixture	*Incorporation (counts/min/mg protein)*	
	Mitochondria	*Microsomes*
Complete system	168	656
Minus ATP-regenerating system	15	80
Minus cell sap	144	110
Plus mixture of nucleoside triphosphates	288	640
Plus RNA-ase	165	20
Plus chloramphenicol	39	649
Plus chlortetracycline	22	624

TABLE II

INHIBITION BY CHLORAMPHENICOL AND CHLORTETRACYCLINE OF AMINO ACID UPTAKE BY PROTEINS OF THE MITOCHONDRIAL SYSTEM

Complete system and conditions essentially as in Table I. All experiments run in duplicate and zero-time controls included for each combination.

Addition to complete system	*Incorporation (counts/min/mg protein)*	
	Standard assay	*Hot extraction omitted*
None	212	208
Chloramphenicol	38	148
Chlortetracycline	25	106

Experiments done with chlortetracycline, under conditions similar to those reported for chloramphenicol, yielded strictly analogous results.

Further characterization of the compound accumulating in the presence of chloramphenicol and chlortetracycline may aid in locating the exact site of action of these antibiotics and may also contribute towards the elucidation of the mechanism involved in the amino acid uptake by the mitochondrial system.

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Inhibition of growth* of cultured mammalian cells by liver extracts

In spite of the genotypic capabilities of at least some of their cells, in adult animals, cell multiplication is largely suppressed and occurs only at a rate sufficient to replace dead ones. Two obvious mechanisms may be invoked to explain this steady state: the existence of one or more growth-limiting factors for each cell type (a series of chemostat-like controls) and the synthesis of growth inhibitors to restrain themselves or other cells.

Because of the advantages they provide over the whole animal, evidence for the existence of growth inhibitors was sought with cultured cells. To this end, the effects were studied of various mature rabbit organ extracts and of serum upon the multiplication of liver and kidney cells freshly cultured from 4-week old rabbits.

Serum (up to 50%) and extracts of kidney, spleen, and lung (about 0.5 mg protein/ml) were not inhibitory. Liver extracts, on the other hand, not only depressed or completely prevented the growth of the liver and kidney cells but of an established line of fibroblast-like mammalian cells (AMK 2-2) as well. Measured after 4 or 5 days of incubation, cell yields with each of the cultures were depressed about 50% with 0.02 mg liver protein/ml and growth was completely prevented when 0.09 mg protein was added/ml of growth medium.

The inhibitory effect of liver preparations appears to result from their content of arginase which depletes the medium of an amino acid essential for growth. The evidence in support of this contention is as follows: (a) the inhibitor is destroyed by heat (100°, 2 min); (b) it appears to be present only in liver; and (c) of the compounds tested, including all the amino acids (0.003 *M* except for tryptophan and methionine (each 0.001 *M*)) and vitamins of the basal growth medium[2], only arginine, citrulline, and ornithine prevented the inhibitory action of liver extracts. Thus, with the AMK cells, in the presence of 0.09 mg liver protein/ml, no inhibition of growth occurred upon the addition of $5 \cdot 10^{-3}$ *M* L-arginine, $2 \cdot 10^{-4}$ *M* L-citrulline, or $7 \cdot 10^{-3}$ *M* L-ornithine. Similar results were obtained with liver cells** except that a higher concentration of citrulline ($3 \cdot 10^{-3}$ *M*) was required for a maximal effect. Whether ornithine serves as a precursor of arginine or merely inhibits arginase is not known. No evidence could be found for the presence of additional growth inhibitors in liver extracts.

While they shed no light on growth controls *in vivo*, these results indicate one mechanism of toxicity of crude biologic preparations for cultured animal cells. In addition, they suggest that caution should be used in interpreting the results of growth-inhibitor experiments involving the injection of organ extracts into animals.

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* Growth is used to describe an increase in cell numbers.
** With kidney cells, the three amino acids were tested at only one level.

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Volumes of distribution of hemoglobin, of [^{14}C]carboxypolyglucose and of [^{14}C]sucrose in pellets of rat-liver mitochondria

In order to determine the concentration of various solutes within the mitochondria, it is usually necessary to estimate and make an appropriate correction for the interspace of the mitochondrial pellet. The [^{131}I]albumin space[1] has been used as a measure of the extraparticulate volume and yields values of about 18 % of the total pellet volume. However, it was concluded that a significant fraction of the iodinated albumin was bound by the mitochondria. WERKHEISER AND BARTLEY[2] used the volume of distribution of [^{14}C]carboxypolyglucose, a branch-chained glucose polymer with a molecular weight of approximately 50,000, as a measure of the interspace in mitochondrial pellets. They reported this space to be 19–22 % of the total pellet water. In subsequent work, AMOORE AND BARTLEY[3] found carboxypolyglucose spaces ranging from 25–54 % of the total pellet water. These investigators[2,3] have also reported that a large fraction of the intramitochondrial water is available to sucrose. These latter observations have been confirmed by MALAMED AND RECKNAGEL[4]. Since polyglucose is not readily available, we have sought some other compound which could be used to estimate the extraparticulate volume of mitochondrial pellets. Thus, a comparison was made of the volumes of distribution of three large molecular weight compounds, hemoglobin, [^{14}C]carboxypolyglucose and [^{14}C]carboxydextran, in pellets of rat-liver mitochondria. The [^{14}C]sucrose space was also measured.

Mitochondria were prepared as previously described[5] from 72 g rat liver in 0.3 M sucrose. The mitochondria were washed once, resuspended in cold 0.3 M sucrose and divided equally among six tared, heavy-walled, 12 ml, Pyrex centrifuge tubes. After centrifugation at $25{,}000 \times g$ for 10 min in the Servall Superspeed refrigerated centrifuge, the supernatants were discarded and the walls of the tubes were wiped dry with strips of filter paper. The mitochondria were resuspended in 0.5 ml 0.3 M sucrose containing known concentrations of [^{14}C]carboxypolyglucose, [^{14}C]carboxydextran, hemoglobin* or uniformly labeled [^{14}C]sucrose. The tubes were then centrifuged at $25{,}000 \times g$ for 10 min. The supernatants were removed for analysis, and the walls of the tubes were again wiped dry with filter paper. All the above procedures were carried out in the cold. After the mitochondrial pellets were weighed, they were dried overnight in a vacuum oven at 105–110°. The volume of distribution of each compound was calculated from its dilution in the supernatant. The water content of the mitochondrial pellets was estimated from the differences between their wet and dry weights. Hemoglobin was estimated by the benzidine method of BING AND BAKER[6] as modified by HAM[7]. The polyglucose and dextran were made radioactive by the addition of [^{14}C]carboxyl groups to the reducing ends of the chains[2,8]. The dextran** was not homogeneous, but was a mixture of macromolecular material having an average molecular weight approximating that of serum albumin; the molecular weights ranged from 25,000 to 200,000.

In two experiments the volume of distribution of hemoglobin in the mitochondrial pellets varied between 4,000 and 10,000 times the total pellet water. It was concluded that the hemoglobin was extensively adsorbed to the mitochondria, and no further experiments were performed with this substance. The volumes of distribution of $[^{14}C]$carboxypolyglucose, of $[^{14}C]$carboxydextran and of $[^{14}C]$sucrose are summarized in Table I. The sucrose and carboxypolyglucose spaces were 61.0 % and 29.8 %, respectively, of the total pellet water. These values are in good agreement with those obtained by WERKHEISER AND BARTLEY[2] and AMOORE AND BARTLEY[3]. The volume of distribution of the carboxydextran was identical to that of the carboxypolyglucose, indicating that these two compounds can be used interchangeably.

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* Purified (twice recrystallized) bovine hemoglobin, supplied by the Nutritional Biochemicals Corp.

** An aliquot of a 6% (w/v) dextran solution in 0.9% NaCl (Plavolex, Wyeth), used for intravenous infusion, was dialyzed against running tap water for 3 days to remove the salt and preservative.

TABLE I

VOLUMES OF DISTRIBUTION OF $[^{14}C]$SUCROSE, $[^{14}C]$CARBOXYPOLYGLUCOSE AND $[^{14}C]$CARBOXYDEXTRAN IN PELLETS OF RAT-LIVER MITOCHONDRIA

The volumes of distribution are expressed as % of total pellet water.

	Sucrose	*Carboxy-polyglucose*	*Carboxydextran*
Mean	61.0	29.8	29.9
S.E.	6.24	1.37	1.58
No. of samples	6^{*}	10^{**}	8^{**}

* 3 experiments. ** 4 experiments.

There is, presently, no absolute measure of the extraparticulate space of mitochondrial pellets. However, the reasons which suggest that the volumes of distribution of carboxypolyglucose and carboxydextran may be used as an index of the extraparticulate space are summarized as follows: (a) these compounds have high molecular weights; (b) their volumes of distribution are smaller than those of other, smaller molecular weight compounds which have been tested[2,3]; (c) the volumes of distribution of carboxypolyglucose and carboxydextran are of the order of magnitude which one would expect theoretically[9].

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Separation of phosphate esters by paper chromatography

Paper-chromatographic methods have been used extensively for the separation and identification of phosphate esters formed during photosynthesis[1] or from glycolysis of sugars and sugar derivatives by a variety of tissues[2,3]. HANES AND ISHERWOOD[4] made a thorough study of a variety of solvents, papers and conditions for the separation of phosphate esters. Their findings encouraged further studies and this resulted in reports of procedures by BANDURSKY AND AXELROD[5], BENSON *et al.*[6], and MORTIMER[7] which have found wide application.

Some of these procedures require extensive time intervals for resolution of esters, thus increasing diffusion of the spots on paper[1], while others require low temperatures[5]. This report presents a procedure for the separation of phosphate esters at room temperature using ethyl acetate–acetic acid–water[7] in one direction and methyl ethyl ketone–methyl cellosolve–ammonia[7] in the second direction.

Whatman 41 H or Schleicher and Schuell 589 blue ribbon paper was used without pretreatment throughout these studies. Standards were prepared from barium salts of phosphate esters purchased from Schwartz Laboratories, Inc. Barium was removed by treating a 2 % solution of each ester salt with Dowex 50-H^+ (8 % cross-linked 80–100 mesh). When phosphate esters were chromatographed from tissue incubations, it was first necessary to remove the cations in the deproteinized solution (containing trichloroacetic acid or $HClO_4$) by treatment with Dowex 50-H^+. Following this treatment, the centrifuged supernatant was used for paper chromatography. The methyl ethyl ketone solvent mixture, prepared from commercially available solvents, required pretreatment before use in order to remove unidentified impurities which caused rapid deterioration of the solvent mixture during chromatography. 7 Parts (by vol.) of methyl ethyl ketone were mixed with 2 parts of methyl cellosolve and this mixture was filtered through a bed of Dowex 50-H^+ previously drained free of excessive moisture by suction. Subsequently, 0.7 part of concentrated ammonia and 2.3 parts of water were added to 9 parts of the treated solvents, and this preparation was ready for chromatography.

Samples were applied to the paper in three or four 5-µl aliquots, the spots being dried between aliquots with a cold air blower. The chromatograms were first developed in an ascending direction with ethyl acetate–acetic acid–water (3:3:1, v/v/v) for 15 h, then dried, and developed in a descending direction at right angle with methyl ethyl ketone–methyl cellosolve–concentrated ammonia–water (7:2:0.7:2.3, v/v/v/v) for 22 h during which the solvent was permitted to drip from a serrated edge. The papers were dried and the phosphate esters located by one or a combination of the following spray reagents:

1. Acid molybdate spray was used to locate phosphate ions according to the procedure described by HANES AND ISHERWOOD[4]. Before spraying with this reagent it was necessary to spray the developed chromatogram with 10 % trichloroacetic acid and allow the paper to hang for 1 or 2 h to facilitate hydrolysis, particularly of glycerol phosphate and diphosphoglyceric acid.

Abbreviations: FDP, fructose diphosphate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; DPG, 2,3-diphosphoglyceric acid; 2PG, 2-phosphoglyceric acid; 3PG, 3-phosphoglyceric acid; GP, glycerol phosphate; R5P, ribose-5-phosphate; HP, hexose phosphates; P–P, polyphosphate esters; iP, inorganic orthophosphate.

2. For detection of carbohydrates, a 0.6 % ethanol solution of *p*-aminohippuric acid was used containing 0.3 % trichloroacetic acid[8]. The sprayed papers were then heated at 150° for 2–3 min, and the carbohydrates were located as fluorescent spots under an u.v. light. The sensitivity of this method is approximately 0.5 μg.

When this procedure was used for the separation of [^{32}P]phosphate esters from tissue incubations, autoradiograms were made by direct contact of dry paper chromatograms (before spraying with any reagents) with no-screen X-ray film for 48 h. The esters were then located by means of the developed X-ray, cut from the paper and pasted on metal or cardboard discs for radioactivity determinations with an end-window Geiger Müller tube.

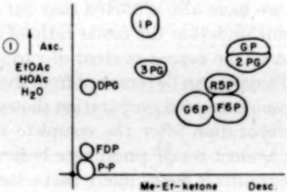

Fig. 1. Diagramatic representation of a two-dimensional separation of phosphate esters by paper chromatography.

Fig. 1 is a diagrammatic drawing of a two-dimensional chromatogram illustrating the location and separation of phosphate esters tested. This method of two-dimensional chromatography permits separation of FDP, DPG, 3PG, and polyphosphates (mainly coenzymes). Some esters, G6P and F6P, GP and 2PG, show some overlapping. This procedure gives good reproducible chromatograms under the stated conditions, and with ^{32}P-labeled esters allows quantitative evaluation of isotope distribution among the separated esters.

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Properties of copper in purified cytochrome *a*

EICHEL *et al.*[1] and GREEN AND CRANE[2] have reported that their cytochrome oxidase preparations contain a high concentration of copper as well as heme iron. On the other hand, many investigators[3–5] have observed from dietary experiments that copper-deficient tissues had a low cytochrome oxidase activity and a decreased content of heme *a*. Based on the above results, it has been considered that the copper in the cytochrome oxidase of typical cytochrome systems plays an important role in the mechanism of the cytochrome oxidase reaction. However, there is no direct evidence to support the above role of copper. The present communication deals with some properties of copper in the preparation of cytochrome *a* purified by our method[6].

In a previous report[6], we have also reported that our cytochrome *a* preparation contains copper in an amount such that the molar ratio of copper to heme iron is 4.5. Later, we repeated analyses of the copper content of various preparations and found that about 80 % of the total copper can be removed from the protein moiety by dialysis against cyanide, but the cyanide-treated preparation shows the same oxidase activity as that of an untreated preparation after the complete removal of cyanide by re-dialysis of the preparation against 0.1 *M* phosphate buffer containing 0.5 % Emasol 4130 at pH 7.4. From these results it seems likely that a large part of the total copper is not required for oxidase activity, and it may be adsorbed on the protein during the purification. In order to determine whether or not this is true, cytochrome *a* was prepared according to our method by using water from which copper was removed, and the copper content of the preparation thus obtained was determined. As illustrated in Table I, five samples of Fraction S_4 of cytochrome *a* contain equimolar amounts of copper and cytochrome *a* or iron. The relation between cytochrome *a* and copper contents during the purification process are also illustrated in Table II. All the results indicate that the molar ratio of copper to cytochrome *a* is always 1. About 50 % of the cytochrome oxidase activity is inhibited by copper-chelating agents such as ferrocyanide, salicylaldoxime, or ethylxanthate as illustrated in Table III. However, the copper in cytochrome *a* is not released by dialysis against cyanide or salicyladoxime and the activity is not altered by the treatment as described the above. The activity after the above treatment is also inhibited by the above copper-chelating agents at

TABLE I

COPPER AND IRON CONTENTS IN FRACTION S_4 OF CYTOCHROME *a*

Preparation No.	*Contents (10^{-3} µatoms or µmoles/mg protein N)*			$\frac{Cu}{Fe}$	$\frac{Cu}{Cyt.\ a}$
	*Cu**	*Fe***	*Cyt. a****		
1	6.35	6.07	5.65	1.05	1.12
2	5.68	5.65	4.91	1.01	1.16
3	5.91	5.65	5.36	1.04	1.10
4	5.58	4.89	4.23	1.10	1.32
5	6.27	5.95	5.09	1.05	1.23
Average	5.96	5.64	5.05	1.05	1.18

* The copper content was determined by the method of MCFARLANE[8].

** The iron content was determined by the method of SANDELL[9].

*** The concentration of cytochrome *a* was determined spectrophotometrically assuming[10] that $\varepsilon^{(\text{reduced})}_{605-630\ \text{m}\mu} = 16.5 \cdot 10^3\ M/\text{cm}$.

TABLE II

RELATION BETWEEN COPPER AND CYTOCHROME *a* CONTENTS DURING THE PURIDICATION PROCESS

*Stage of purification**	S_1	S_2	S_3	S_4
Turnover number** (μmoles O_2/μmole cyt. *a*/min)	114.2	150.0	42.2	36.4
Cytochrome a (10^{-2} μatom/mg N)	1.05	1.07	3.75	5.09
Copper (10^{-2} μatom/mg N)	1.10	1.43	4.76	6.09
Copper/cytochrome *a*	1.04	1.33	1.27	1.19

* The nomenclature of the fractions is the same in a previous report[6].
** The activity was measured manometrically in 1 % Emasol 4130[10].

TABLE III

EFFECT OF COPPER-CHELATING AGENTS ON THE ACTIVITY OF CYTOCHROME *a*

Reagent	*Concentration (M)*	*Inhibition* (%)*
KCN	10^{-5}	60
	10^{-4}	100
NaN_3	10^{-3}	72
$K_4Fe(CN)_6$	10^{-2}	50
Salicylaldoxime	10^{-2}	50
Ethylxanthate	10^{-2}	58

* The activity was measured manometrically at 30° by using hydroquinone as substrate. Fraction S_4 was used as purified cytochrome *a*.

the same ratio. Acid treatment of cytochrome *a* can easily release the copper from the protein moiety, while heat treatment cannot even at 100° for 30 min.

To check the valency state of copper in cytochrome *a*, color development by the addition of 2,2′-diquinolyl or 2,9-dimethyl-1,10-phenanthroline was also examined. By the addition of either of the above reagents, cytochrome *a* in Emasol 4130 strongly exhibits the specific color due to Cu^+. However, as in the case of iron in succinic dehydrogenase[7], the color is not developed in the presence of *p*-chloromercuribenzoate. Based on these results, it appears that the copper in cytochrome *a* exists as in the cupric state. Cytochrome *a* in its oxidized form shows a spectrum having absorption maxima at 280, 340, 424, and 600 mμ. Absorbancy at the 340 mμ peak is decreased by dialysis against cyanide and recovered by the addition of Cu^{++} but not by the addition of Cu^+. However, there is no direct evidence that the 340 mμ peak is due to the combination of cytochrome *a* with copper. We are now studying the valency change of copper in cytochrome *a* in the presence of reduced or oxidized cytochrome *c* to investigate the possibility that the copper can act as an electron carrier. The results of these studies will be presented elsewhere.

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Studies on the electron transport system

XIV. The isolation and properties of soluble cytochrome c_1. Addendum

Since the publication of the paper on the isolation and properties of cytochrome c_1[1] it has been pointed out to the authors[2] that the calculations of the oxidation–reduction potential of cytochrome c_1 were not based on the most reliable E_0 value for the benzoquinone–hydroquinone system (see p. 44 and Table VI in GREEN *et al.*[1]). When the more correct value of + 0.69607 V[3] is used for the E_0 of the reference quinone and 0.060148 for the factor 2.30258 RT/F, the mid-point potentials for cytochromes c_1 and c shown in the accompanying table are obtained. This table is a reproduction of our original table, but with the revised values substituted for the earlier ones. The mean E_0' for the cytochrome c_1 system is + 0.220 V, and for the cytochrome c system + 0.248 V. This latter value has to be compared with E_0' values obtained by others, which range from + 0.254 V to + 0.262 V.

TABLE I

DETERMINATION OF THE OXIDATION-REDUCTION POTENTIAL OF CYTOCHROMES c_1 AND c

The measurements were made in 0.1 M phosphate at 30°. The *p*-benzoquinone and hydroquinone solutions were prepared in ethanol from recrystallized samples.

Concentration of quinone-hydroxyquinone mixture (µmoles/ml)	*Cytochrome*	*pH*	*E_0' of quinone system (V)*	*Total concentration of hemoprotein (µmoles/ml)*	*Final concentration of hemoprotein*		*$0.060148 \times \log \frac{ox}{red}$*	*E_0' (V)*
					oxidized (µmoles/ml)	*reduced (µmoles/ml)*		
1	c_1	6.73	+ 0.2913	0.034	0.0314	0.0026	0.0651	+ 0.226
1	c_1	7.55	+ 0.2419	0.034	0.026	0.008	0.0308	+ 0.211
0.5	c_1	7.55	+ 0.2419	0.034	0.023	0.011	0.0193	+ 0.223
6	c	6.73	+ 0.2913	0.105	0.081	0.024	0.0321	+ 0.259
6	c	7.55	+ 0.2419	0.105	0.058	0.047	0.0055	+ 0.236

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Observations on the reactivation of isooctane-extracted DPNH-cytochrome *c* reductase with D-[^{14}C]-α-tocopherol

Previous experiments[1,2] have demonstrated that after administration of [5-Me-^{14}C]-D-α-tocopheryl succinate to rats, two radioactive metabolites, in addition to α-tocopherol, are extractable from the liver. One of these ("Compound F") exhibits an absorption maximum at 257 mμ and is reducible to α-tocopherol with $SnCl_2$ in HCl. The other ("Compound O") has an absorption maximum at 255 mμ and is associated predominantly with the microsomes. Evidence that the DPNH-cytochrome *c* reductases of rat skeletal muscle and bovine heart muscle can be inactivated by isooctane extraction or aging, and restored specifically by α-tocopherol[3,4], prompted a study of the metabolism of [^{14}C]-α-tocopherol by similar preparations of the electron-transport system.

Keilin-Hartree heart-muscle preparations containing about 15 mg protein/ml were used[5]. Extraction was carried out by shaking 5 times for 15 min with 5 vol. isooctane at 4°. After each extraction the preparation was centrifuged for 2 min at 500 × *g* and the isooctane layer was removed. The resulting fine emulsion, which contained an active DPNH-cytochrome *c* reductase, could be partially inactivated by centrifuging for 2 h at 15,000 × *g* in the refrigerated centrifuge, and further inactivated by another centrifugation at 30,000 × *g* for 2 h, the clear isooctane layer being removed each time and the remainder resuspended in a hand homogenizer.

DPNH-cytochrome *c* reductase was measured spectrophotometrically at 550 mμ using a 3.5-ml reaction mixture similar to that of DEUL *et al.*[6]: heart-muscle preparation, 0.1 mg protein/ml; cytochrome *c*, $2.8 \cdot 10^{-5}\,M$; DPNH, $1.8 \cdot 10^{-4}\,M$; KCN, $10^{-3}\,M$; EDTA, $10^{-3}\,M$; phosphate buffer, pH 7.3, 0.033 *M*. DPNH oxidase was measured at 340 mμ with KCN omitted. *d*-α-tocopherol was added at $10^{-4}\,M$ as a suspension in 0.2 % serum albumin[6].

After testing for reactivation with unlabelled tocopherol, the enzyme preparation was regenerated with D-[5-Me-^{14}C]-α-tocopherol ($10^{-4}\,M$, 8,100 disintegrations/min/7 ml reaction mixture). A parallel experiment was carried out using an unextracted heart-muscle preparation. Following a 30-min reaction period the mixture was extracted 3 times with peroxide-free ether which, after backwashing with water, was concentrated *in vacuo* under N_2 at 50°. The residue was applied in ethanol to three different paper chromatographic systems which are suitable for the separation of metabolites of α-tocopherol formed *in vivo*[1,2]. Scanograms were prepared from the developed chromatograms for the determination of R_F values.

The radioactivity in the ether extract of both the reactivated DPNH-cytochrome *c* reductase and the unextracted enzyme was due entirely to unchanged D-[^{14}C]-α-tocopherol. Isooctane extraction partially inactivated DPNH oxidase, but no appreciable reactivation was achieved with α-tocopherol. Incubation of labelled tocopherol with unextracted heart-muscle preparation in the DPNH oxidase system also failed to yield a labelled metabolite.

The results demonstrate that the reactivation of isooctane-extracted DPNH-cytochrome *c* reductase is not accompanied by enzymic oxidation of α-tocopherol and is probably due to a physical effect upon residual isooctane in the system, as

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Abbreviations: DPNH, reduced diphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetic acid; DPPD, N,N′-diphenyl-*p*-phenylenediamine.

suggested elsewhere[6]. The effectiveness of various isoprenoid compounds in restoring isooctane-extracted systems has been demonstrated by other workers[6–8].

Experiments with rabbits and rats in this laboratory have provided strong evidence for the direct substitution of α-tocopherol by the synthetic antioxidant DPPD in functions relating to the two classical symptoms of vitamin E deficiency, namely, muscular dystrophy and resorption gestation[9,10]. Accordingly, it was of interest to determine whether this compound could replace α-tocopherol in the regeneration of the isooctane-extracted DPNH-cytochrome *c* reductase. Santoquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline), another synthetic antioxidant which has been shown to be effective in preventing vitamin E deficiency in animals, was also tested. The results, shown in Table I, demonstrate that both antioxidants are effective in regenerating the extracted enzyme in molar concentrations comparable to, or less than, that required for α-tocopherol. Similar observations have been made by other workers[11]. In agreement with previous reports[6–8], reactivation was also obtained with ubiquinone (10^{-4} *M*) and vitamin K_1 (10^{-3} *M*).

TABLE I

INFLUENCE OF ANTIOXIDANTS ON ISOOCTANE-EXTRACTED DPNH-CYTOCHROME *c* REDUCTASE

	Relative activity
Unextracted heart-muscle preparation	100
Extracted heart-muscle preparation	25
Extracted heart-muscle preparation + DPPD ($5.5 \cdot 10^{-4}$ *M*)	32
Extracted heart-muscle preparation + DPPD ($1.1 \cdot 10^{-5}$ *M*)	33
Extracted heart-muscle preparation + DPPD ($5.5 \cdot 10^{-5}$ *M*)	89
Extracted heart-muscle preparation + Santoquin ($6.2 \cdot 10^{-5}$ *M*)	71
Extracted heart-muscle preparation + D-α-tocopherol (10^{-4} *M*)	75

See text for composition of reaction mixture. Antioxidants and α-tocopherol added in 10 µl ethanol.

These findings do not support the suggestion[7,8] that a necessary criterion for regeneration of the isooctane-treated enzyme is a long isoprenoid side chain. The possibility that reactivation is dependent upon antioxidant activity, on the other hand, is also remote, as compounds without such activity are effective and since [^{14}C]-α-tocopherol undergoes no oxidative change coincident with reactivation of the system. A purely physical mechanism, such as isooctane dispersion on the enzyme[6], is therefore indicated.

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Beschleunigung des oxydativen Pentosephosphatcyclus in Hefezellen durch Ammoniumsalze

GLOCK UND MCLEAN zeigten, dass in verschiedensten tierischen Geweben das Wasserstoff-übertragende Coenzym TPN überwiegend in reduzierter Form vorliegt[1]. Dasselbe gilt für Glucose oxydierende Bäckerhefezellen: mit einer früher beschriebenen Methode[2] fanden wir pro Gramm Frischgewicht 10 μg TPN neben 46 μg TPNH. In Hefe überwiegen demnach wie in anderen Zellen und Geweben die TPN reduzierenden Reaktionen über die TPNH oxydierenden Reaktionen. Man konnte aufgrund dieser Situation vermuten, dass der auf Vorhandensein von oxydiertem TPN angewiesene oxydative Pentosephosphatcyclus durch Mangel an TPN limitiert ist und dass durch Ingangsetzung zusätzlicher, TPNH oxydierender Systeme eine Beschleunigung des Glucoseabbaues über den oxydativen Pentosephosphatcyclus bewirkt werden kann (Zusammenfassung siehe[3,4]). Tatsächlich beobachteten KINOSHITA[5] an Retina nach Zusatz von Pyruvat, HERS[6] an Leberschnitten nach Zusatz von Glucoson, Glucuronolacton oder Glycerinaldehyd, CAHILL *et al.*[7] an Leberschnitten nach Zusatz von Methylenblau oder Pyocyanin und BRIN UND YONEMOTO[8] nach Zusatz von Methylenblau zu Erythrocyten eine Beschleunigung des Pentosephosphatcyclus. In allen Fällen bewirkten die zugesetzten Metaboliten oder Farbstoffe eine vermehrte Oxydation von TPNH zu TPN.

In früheren Versuchen fanden wir Anhaltspunkte dafür, dass nach Zusatz von Ammonium-Ionen zu Glucose oxydierenden Hefezellen durch Einsetzen der TPNH verbrauchenden reduktiven Aminierung von α-Ketoglutarat zu Glutaminsäure eine Verschiebung des Glucoseabbaues vom Weg über Glycolyse und Citronensäurecyclus zum Weg des oxydativen Pentosephosphatcyclus stattfindet[9-12]. Insbesondere spricht die nach Ammoniumzusatz beobachtete Abnahme der Glucose-6-phosphat-Konzentration und die gleichzeitig damit stattfindende Zunahme der Konzentration an oxydiertem TPN[10] für eine solche Verschiebung.

TABELLE I

ISOTOPENANALYSE DES BEIM AEROBEN UMSATZ VON [1-C^{14}]GLUCOSE UND [6-^{14}C]GLUCOSE IN HEFANSÄTZEN MIT UND OHNE AMMONIUMSULFAT ENTWICKELTEN CO_2

Pro Warburggefäss (Gesamtvolumen der Ansätze 2.0 ml; 0.083 *M* Citratpuffer pH 6.0; 0.2 % Glucose; ± 0.08 *M* $(NH_4)_2SO_4$; Gasphase Luft; im Einsatz 0.2 ml 2 *N* NaOH) wurden 15 mg gewaschene und verarmte Bäckerhefe der Sinner AG. (Karlsruhe) mit 1 μC [1-^{14}C]Glucose bzw. [6-^{14}C]Glucose 50 min bei 25° inkubiert. Fällung des CO_2 aus dem NaOH-Einsatz nach Zusatz von inaktivem Na_2CO_3 als $BaCO_3$. Auszählung bei "unendlicher Schichtdicke" (Massenbelegung 12 mg/cm^2) im fensterlosen Methandurchflusszähler der Firma Friesecke und Höpfner (FHZ 407) bis zur 1.5 % Fehlergrenze.

	Versuch	*$^{14}CO_2$ aus [1-^{14}C]Glucose (Impulse/min/ Warburg-Gefäss)*	*$^{14}CO_2$ aus [6-^{14}C]Glucose (Impulse/min/ Warburg-Gefäss)*	*C_1/C_6*
Ansatz mit NH_4^+	1	9,600	1,450	6.6
	2	11,080	1,730	6.4
Ansatz ohne NH_4^+	1	2,800	1,170	2.4
	2	3,170	1,260	2.5

Abkürzungen: TPN bzw. TPNH, oxydiertes bzw. reduziertes Triphosphopyridinnucleotid; DPN, Diphosphopyridinnucleotid.

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Versuche mit Glucose, die an C_1 bzw. C_6 mit ^{14}C markiert war, beweisen nun die von uns angenommene Verschiebung des Glucose-Abbaues in Hefezellen. Man sieht aus Tabelle I, dass der Zusatz von NH_4^+ zu Glucose oxydierender Hefe die $^{14}CO_2$-Freisetzung um das 2.5 fache zugunsten von [1-^{14}C]Glucose verschiebt. Dies spricht eindeutig für eine Intensivierung des Glucose-Abbaues über den oxydativen Pentosephosphatcyclus nach Zusatz von Ammoniumsalzen. Die durch NH_4^+-Zusatz ausgelöste Oxydation von TPNH zu TPN findet wahrscheinlich durch 2 Mechanismen statt: (a) Reduktive Aminierung von α-Ketoglutarat zu Glutaminsäure, (b) Wasserstoffübertragung von TPNH auf DPN durch Zusammenwirken der in Hefe nachgewiesenen DPN-spezifischen und TPN-spezifischen Glutaminsäuredehydrogenasen[9]. Hefezellen, die in Phosphatpuffer glucose oxydieren, erhalten mit dem Angebot von Ammoniumsalzen die entscheidende stoffliche Voraussetzung für Wachstum und Zellvermehrung. Deshalb liegt in der nach Ammonium-Zusatz einsetzenden vermehrten Produktion von Pentosephosphaten aus Glucose ein "sinnvoller" Mechanismus vor: Pentosephosphate werden zur Synthese von Nucleinsäuren benötigt und stellen damit wichtige, für Wachstum und Zellvermehrung benötigte, Stoffwechselzwischenprodukte dar.

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Activation of methionine for transmethylation

IV. The failure of 3,5′-cycloadenosine to replace adenosine triphosphate[*]

The enzymic activation of methionine for transmethylation is known to involve the formation of a sulfonium compound[1], (—)-S-adenosyl-L-methionine[**], by a reaction

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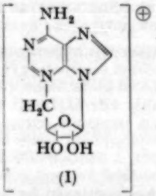
Abbreviations: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate.

[*] Paper No. 17 in a series on enzymic mechanisms in transmethylation.

[**] In the term (—)-S-adenosyl-L-methionine the L refers to the configuration of the α-amino carbon atom while the (—)refers to the contribution of the asymmetric sulfonium center to the net rotation of the molecule. This terminology is based upon the finding that only one sulfonium diastereoisomer is formed enzymically and is active as a transalkylating agent with several enzyme systems[2].

in which the terminal phosphate group of ATP is released as orthophosphate and the proximal two are released as pyrophosphate[3,4].

Earlier studies on the mechanism of this reaction led to the suggestion that it might proceed stepwise with the intermediate formation of a 3,5′-*cyclo*adenosine cation (I) which would undergo subsequent nucleophilic attack by the sulfur atom of methionine[3].



(I)

Although this sequence is only one of a number of possible alternatives, it was of interest to attempt the synthesis of the *cyclo* compound as a means of exploring further the mechanism of the enzymic reaction.

Although 3,5′-*cyclo*adenosine could be prepared from 2′,3′-*iso*propylidene-3,5′-*cyclo*adenosine[5] by acid hydrolysis, the conditions required for the removal of the protecting group (0.1 *M* HCl at 100° for 45 min) led to a complex mixture of products. A more satisfactory synthesis involved the removal of the *iso*propylidene group from 2′,3′-*iso*propylidene-5′-*p*-toluenesulfonyl adenosine[5,6] by treatment with formic acid saturated with HCl at ice-bath temperature for 30 min and subsequent cyclisation in boiling water. The ionic compound was then isolated as the reineckate, converted to the chloride salt, and repeatedly crystallised from aq. alcohol. 3,5′-*cyclo*Adenosine chloride (Found: C, 41.69; H, 4.37; Cl, 12.32; N, 24.77; calcd. for $C_{10}H_{12}O_3N_5Cl$: C, 41.59; H, 4.15; Cl, 12.31; N, 24.25) absorbed u.v. light maximally at 272 mμ, moved rapidly towards the cathode on paper electrophoresis and was susceptible to periodate oxidation. It decomposed, without melting, above 230°.

To test the suggestion that 3,5′-*cyclo*adenosine cation might be an intermediate in the enzymic activation of methionine, this compound has been tested as a precursor of S-adenosylmethionine using the methionine-activating enzymes of both liver and yeast.

In a preliminary experiment yeast enzyme was incubated in reaction mixtures containing [2-^{14}C]methionine, K^+, Mg^{++} and buffer, but with 3,5′-*cyclo*adenosine chloride (3.1 or 12.6 μmoles/ml) in lieu of ATP. After incubation, carrier S-adenosylmethionine was added and reisolated chromatographically[7]. In neither case was there an increase in the radioactivity of the column fraction containing S-adenosylmethionine over that found in the control from which methionine was omitted. From a parallel experiment using ATP (24.4 m*M*), it was calculated that formation of S-adenosylmethionine from the *cyclo*nucleoside at 10% of the rate found with ATP could have been readily detected. Similar results were obtained in a corresponding experiment using the methionine-activating enzyme of rabbit liver.

Although 3,5′-*cyclo*adenosine itself did not appear to be an intermediate in the activation reaction a second type of experiment was carried out to investigate the possible formation of an intermediate which could break down to the *cyclo*nucleoside or to certain other compounds. In this experiment [8-^{14}C]adenosine triphosphate was used as substrate. Non-radioactive pools of 3,5′-*cyclo*adenosine, adenosine, and ADP were included in the reaction mixture and at the end of the incubation with enzyme were reisolated and assayed for radioactivity. To increase the chances of promoting a

TABLE I

RADIOACTIVITY OF NUCLEOSIDES ISOLATED AFTER INCUBATION OF [8-^{14}C]ATP WITH METHIONINE-ACTIVATING ENZYME OF YEAST

All vessels contained (in μmoles): either tris(hydroxymethyl)aminomethane buffer, pH 7.5, 50, or imidazole buffer, pH 5.6, 50 and sodium succinate, pH 5.6, 25. The complete reaction mixtures contained: KCl, 100; $MgCl_2$, 100; reduced glutathione, pH 6.7, 8; L-methionine, 22.5; adenosine, 2.65; 3,5′-*cyclo*adenosine (chloride salt), 2.6; ADP, pH 5.6 or 7.5, 2.4; [8-^{14}C]ATP, pH 6.7, 0.82 (containing $126 \cdot 10^3$ counts/min); yeast inorganic pyrophosphatase, alcohol fraction of HEPPEL[8] sufficient to hydrolyze 40 μmoles pyrophosphate under these conditions. Yeast methionine-activating enzyme, 5.9 units of an $(NH_4)_2SO_4$ A fraction[4]. Omissions were as indicated. Pyrophosphate, where present, 1 μmole (Na salt). The final vol. was 0.78 ml. The reaction was initiated by the addition of enzyme; the incubation was continued for 30 min at 37° and 1.2 ml 6 % trichloroacetic acid was added to stop the reaction. Adenosine 5′-phosphate (2.5 μmoles), ATP (6.8 μmoles), and S-adenosylmethionine (2 μmoles) were added as carriers. The mixture was centrifuged to remove the precipitated protein and the supernatant fluid extracted 3 times with several volumes of ethyl ether. The aqueous phase was neutralized and fractioned by the use of a Dowex-1-Cl column[9]. The material which was washed through the column by 10 vol. water (adenosine, 3,5′-*cyclo*adenosine, and S-adenosylmethionine) was further fractionated by passage over a column of IRC-50 (XE-64) (6 cm × 1 cm), buffered at pH 6.9. Adenosine was eluted[7] with 10 ml 0.01 *M* KPO_4, pH 6.9. Most of the 3,5′-*cyclo*nucleoside was eluted with 25 ml 0.25 *N* acetic acid. Elution was continued with this eluting fluid for an additional 25 ml, but this fraction, containing a few per cent of the 3,5′-*cyclo*adenosine and a small amount of the S-adenosylmethionine was discarded. S-Adenosylmethionine was then eluted with 4.0 *N* acetic acid. ADP, adenosine and 3,5′-*cyclo*adenosine were concentrated by adsorption on Norite A and elution with a small volume of 50% aq. ethanol containing 0.3 ml conc. NH_4OH in each 100 ml[10]. The charcoal was removed by filtration and aliquots of the filtrate were used to determine u.v. absorption and radioactivity. Aliquots of the S-adenosylmethionine and ATP fractions were counted without prior concentration. A thin-window, gas flow counter was used. All samples were corrected for self-absorption and for background. The specific activities were determined on the basis of the radioactivity and of the u.v. absorption ($\varepsilon = 15,400$ at 259 mμ for the nucleotides, adenosine and S-adenosylmethionine; $\varepsilon = 12,400$ at 272 mμ for 3,5′-*cyclo*adenosine). The total radioactivity present in 3,5′-*cyclo*adenosine and adenosine could then be calculated since the net amount of these compounds did not change during the incubation. The total S-adenosylmethionine was assumed to be equal to the amount of carrier added after the incubation (2 μmoles) plus the amount estimated to have been synthesized during the reaction (a maximum of 0.8 μmole). This assumption introduces an uncertainty of no more than 20% in the amount of radioactivity in the S-adenosylmethionine. Calculated in this way, the total recovery of radioactivity ranged from 60–80%.

Omission	*pH*	*Total radioactivity (counts/min)*		
		3,5′-cyclo-adenosine	*Adenosine*	*S-adenosyl-methionine*
Enzyme	7.5	0	80	0
K$^+$	7.5	50	200	17,000
Methionine	7.5	10	70	0
Pyrophosphatase (pyrophosphate added)	7.5	50	330	52,000
None	7.5	10	390	84,000
Enzyme	5.6	0	110	0
K$^+$	5.6	0	110	200
Methionine	5.6	20	120	0
Pyrophosphatase (pyrophosphate added)	5.6	20	160	16,000
None	5.6	20	220	44,000

partial reaction and thus accumulating an intermediate, separate incubations were carried out in which either potassium or methionine was omitted from the complete reaction mixture or inorganic pyrophosphate was added in the absence of pyrophosphatase. A control vessel lacked methionine-activating enzyme. In addition, the entire set of incubations was duplicated at pH 5.6. This pH, although below the optimal pH for the enzyme, might be expected to cause cleavage of an acid-labile P–N bond and thus convert at least in part any hypothetical N-phosphorylated intermediate to a compound which would be trapped in one of the pools present.

The results of this experiment are shown in Table I. It may be seen that less than 0.5 % of the total radioactivity appeared in the 3,5'-*cyclo*adenosine or in the adenosine under any of the sets of conditions. These results appear to rule out the participation of either of these compounds as an intermediate and to make such a role for any N-phosphorylated derivative rather unlikely.

The results obtained with the reisolated ADP are less clear due to the possibility that traces of interfering enzymes were present. However the absence of isotopic dilution by the pool of ADP rules out the participation of free ADP as an intermediate in the conversion of ATP to S-adenosylmethionine, thus affording direct confirmation of a similar conclusion reached earlier in studies of the methionine-activating enzyme of rabbit liver[3].

As noted above, intermediate formation of free *cyclo*nucleoside cation was only one of a number of possible alternative reaction mechanisms leading to the formation of S-adenosylmethionine. Other possibilities still under investigation would require migration of the terminal phosphate of ATP to form a modified adenosine triphosphate. Alternatively, if the reaction proceeds via any intermediate, the latter may be visualized as enzyme bound.

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On the mitochondrial oxidation of isocitrate

It has been shown previously[1,2] that addition of DPN restores the aerobic oxidation of isocitrate in preparations of rat-liver mitochondria that have been aged at 30° in a 0.25 M sucrose–0.05 M phosphate medium for a period of 15 to 30 min. Since this type of treatment is known to lead to a release and destruction of mitochondrial nucleotides in general[3,4] and of the mitochondrial DPN in particular[5], it was anticipated that the preparations so-treated were devoid of endogenous TPN as well. This assumption was also supported by the finding[2] that these mitochondria did not decolorize 2,6-dichlorophenolindophenol in the presence of isocitrate unless a catalytic amount of TPN was added. The restoring effect of added DPN on the oxidation of isocitrate has therefore been interpreted[1,2] to mean that rat-liver mitochondria possess a DPN-specific pathway of isocitrate oxidation which is potent enough to account for the rate at which the aerobic oxidation of isocitrate proceeds in the intact mitochondria. A similar conclusion has recently been reached by GRANT AND MONGKOLKUL[6], concerning mitochondria from ox adrenal cortex.

Meanwhile, however, KAPLAN *et al.*[7] pointed out that mitochondria treated in the way described above still may contain traces of endogenous TPN, and more recently PURVIS[8], on the basis of accurate enzymic and fluorometric measurements, concluded that 1-h aging under the above conditions is necessary for a complete destruction of the liver mitochondrial pyridine nucleotides. From measurements of the reaction rates obtained in aged mitochondria for the reduction of 2,6-dichlorophenolindophenol by isocitrate in the presence of DPN, TPN and DPN + TPN, and for the aerobic oxidation of DPNH and TPNH, the latter with and without DPN, PURVIS[8] also concluded that the reaction sequence, TPN-linked isocitric dehydrogenase ⟶ transhydrogenase ⟶ DPNH-oxidase, constitutes virtually the only pathway sufficiently active to account for the observed rate of isocitrate-oxidation in rat liver (and heart) mitochondria.

In view of these conclusions, it was of interest to test, in the first place, the effect of DPN on the aerobic oxidation of isocitrate in rat-liver mitochondria which have been aged for 1 h or longer. Table I summarizes some typical results. As shown in Expt. 1, added DPN was able to restore isocitrate oxidation equally well after 0.5, 1, 1.5, 3 and 8 h aging. Added TPN had, just as previously found[2] with mitochondria aged for 0.5 h, relatively little effect by itself, and no appreciable additional effect when combined with DPN. Beyond 8-h aging, the level of endogenous substrate, which usually appears upon prolonged aging (and part of which can apparently be oxidized without added pyridine nucleotides) became too high to allow any further evaluation of the DPN effect. Citrate (Expt. 2) which, for reasons now being investigated, was oxidized considerably faster than isocitrate under the present conditions, gave a similar picture to that obtained with isocitrate, in that its oxidation could be restored almost completely by the addition of DPN alone after 1-h aging. Upon prolonged aging, the citrate oxidation could no longer be restored to any larger extent by either DPN or TPN, alone or in combination, probably because of a loss of the aconitase activity. Expt. 3 shows that the DPN effect in restoring isocitrate oxidation in mitochondria aged for 1 h was present even when the mitochondria were recentri-

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Abbreviations: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; ATP, adenosine triphosphate.

TABLE I

EFFECTS OF ADDED PYRIDINE NUCLEOTIDES ON THE AEROBIC OXIDATION OF ISOCITRATE AND CITRATE IN AGED PREPARATIONS OF RAT-LIVER MITOCHONDRIA

The mitochondria were prepared[11] and aged[3] as described previously. The test incubations contained per Warburg vessel: mitochondria from 0.5 g wet wt. liver (about 10 mg biuret protein), 20 μmoles Na citrate or Na DL-isocitrate, 50 μmoles K phosphate (pH 7.5), 3 μmoles Na ATP, 8 μmoles $MgCl_2$, 1 μmole $MnCl_2$, 50 μmoles glucose, 50 μl of a stock solution[10] of yeast hexokinase and 125 μmoles sucrose, in a final volume of 2 ml. When indicated DPN and TPN were added in amounts of 1.5 μmoles each. The vessels were incubated at 30° with air as the gas phase and with 0.2 ml 2 *M* KOH in the center wells. The values refer to the O_2 consumption recorded between 5 and 20 min of incubation.

Expt.	*Time of aging h*	*Substrate*	*Added pryidine nucleotide*				*Notes*
			None	*DPN*	*TPN*	*DPN+TPN*	
			O_2 consumption (μatoms)				
1	0	Isocitrate	5.2	—	—	—	
	0.5	Isocitrate	0.1	4.7	1.5	4.8	
	1	Isocitrate	0.0	4.9	1.5	5.4	
	1.5	Isocitrate	0.3	4.7	2.2	4.9	
	3	Isocitrate	0.1	4.7	0.6	5.2	
	8	Isocitrate	1.9	4.9	1.9	3.6	
2	0	Citrate	12.5	—	—	—	
	1	Citrate	0.8	13.8	2.5	—	
	2	Citrate	1.0	2.3	1.7	2.6	
3	1	Isocitrate	0.7	7.7	—	—	
	1	Isocitrate	0.0	6.0	—	—	Recentrifuged after aging
	1	Isocitrate	0.0	5.8	—	—	Aging with dialysis
4	1	Citrate	1.2	9.9	2.7	10.7	
	1	Citrate	—	9'8	—	—	No hexokinase-glucose in test
	1	Citrate	—	9.4	—	—	No hexokinase-glucose-ATP in test
	1	Citrate	—	8.2	—	—	As above, 10^{-4} *M* dinitrophenol added

fuged after aging and resuspended in a new incubation medium, or when the aging was performed with simultaneous dialysis against 20 vol. of sucrose–phosphate medium or 0.05 *M* phosphate. These treatments, which efficiently remove the endogenous substrates appearing during aging, conceivably also promote the removal of the endogenous pyridine nucleotides. Finally, as shown in Expt. 4, the DPN effect was present also when hexokinase, glucose and ATP were omitted from the incubation mixture (the aged mitochondria, though still capable of oxidative phosphorylation[2,5,7,9], apparently no longer possess the obligatory link between respiration and phosphorylation, characteristic of intact mitochondria[10]) or when a phosphorylation-uncoupling concentration of 2,4-dinitrophenol was added to the system. These findings seem to eliminate the possibility that part of the added DPN was converted to TPN by means of ATP, added or generated during respiration.

The data presented above seem to substantiate the validity of the previous postulate[1,2] that rat-liver mitochondria possess a DPN-catalyzed, TPN-independent pathway by which isocitrate (or citrate) can be oxidized aerobically at a rate approaching the observed rate of aerobic oxidation of isocitrate (or citrate) in the intact mitochondria.

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Transhydrogenase and TPNH oxidase activities of rat-liver mitochondria

Because of recent controversies[1–5] as to the transhydrogenase and TPNH oxidase activities of rat-liver mitochondria, and as to the role played by these pathways in the aerobic oxidation of isocitrate, it was of interest to re-examine previous data published from this laboratory[2–3] on this subject. As a test system, that employed by Purvis[4] was chosen, consisting in a measurement of the rate of aerobic oxidation of TPNH and its increment ensuing the addition of DPN. Two types of assay were performed in parallel: (a) the decrease in the concentration of TPNH was followed at 340 $m\mu$ in a recording DK2 Beckman spectrophotometer, and (b) the oxygen consumption was recorded polarographically, be means of the rotating platinum electrode method[6]*. The mitochondria were depleted of endogenous pyridine nucleotides by aging for 1 h[4] in a sucrose–phosphate medium at 30°. The values in Table I show a good agreement between the two types of assay. The mean values, expressed in terms of μmoles TPNH/min/g liver, are 0.05 for the TPNH oxidase and 0.12 for the transhydrogenase. Previous values[2–3], obtained with a slightly different test system, were 0.05 and 0.08, respectively.

The mean values in Table I, converted to μmoles TPNH/min/mg mitochondrial protein (assuming that mitochondria from 1 g liver contain 20 mg protein[3,8]), are 0.0025 for the TPNH oxidase and 0.006 for the transhydrogenase, which markedly differ from those recently reported by Purvis[4] 0.000 for TPNH oxidase, 0.026 for the transhydrogenase. Although the explanation of these discrepancies must await the publication of the experimental details underlying these values in Purvis's paper[4], the following comments may be helpful in clarifying the situation.

The TPNH oxidase activity reported above was fairly consistent from one experiment to another. It could be increased considerably, however, by increasing the concentration of TPNH. Similar observations were made when cytochrome *c* rather than oxygen was used as a terminal oxidant. A 20-fold increase of the TPNH

Abbreviations: DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide; PNH, reduced pyridine nucleotide.

* I am indebted to Dr. H. Baltscheffsky for his kind help with the use of this technique.

TABLE I

SPECTROPHOTOMETRIC AND POLAROGRAPHIC MEASUREMENTS OF THE AEROBIC OXIDATION OF REDUCED PYRIDINE NUCLEOTIDES (PNH) IN AGED PREPARATIONS OF RAT-LIVER MITOCHONDRIA

Mitochondria, prepared as described elsewhere[24], were aged for 1 h at 30° in a medium of 0.225 *M* sucrose–0.05 *M* phosphate (pH 7.5). The aging suspension contained mitochondria from 0.5 g wet wt. liver (about 10 mg biuret protein)/ml. PNH disappearance was followed at 340 mμ in a recording DK2 Beckman Spectrophotometer, and O_2 consumption was measured polarographically with the rotating platinum electrode method[8]. The test system contained 0.05 *M* K phosphate (pH 7.5) and, where indicated, 0.15 m*M* DPNH or TPNH and 0.5 m*M* DPN or TPN. The samples for spectrophotometry contained mitochondria from 0.1 g liver and had a final volume of 3 ml, and those for polarography contained mitochondria from 0.5 g liver and had a final volume of 1.1 ml. The temperature was about 25°. The reaction rates were constant in both types of assay for at least 5 min in test systems (*d*) and (*f*),and for 1 to 3 min in test system (*b*). The values are expressed in terms of 1 g wet weight liver and minute.

Added pyridine nucleotide	*μmoles PNH oxidized*	*μatoms O_2 consumed*
(*a*) None	—	0.000
(*b*) DPNH	0.465	0.548
(*c*) DPN	—	0.036
(*d*) TPNH	0.044	0.052
(*e*) TPN	—	0.000
(*f*) TPNH + DPN	0.175	0.198
Calculated reaction rates		
DPNH oxidase (*b*)-(*c*)	0.465	0.512
TPNH oxidase (*d*)-(*e*)	0.044	0.052
Transhydrogenase (*f*)-(*e*)-(*d*)-(*c*)	0.131	0.110

concentration, for example, caused a 6- to 8-fold increase of the initial rate of oxidation. As reported previously[9], treatment of mitochondria with 0.05 %–0.1 % deoxycholate results in a similar effect, causing a 6- to 8-fold increase of the TPNH cytochrome *c* reductase and TPNH diaphorase activities as measured with low concentrations of TPNH. The same treatment inactivates the transhydrogenase, in accordance with the earlier observation of KAPLAN *et al.*[10] that this enzyme is sensitive to bile salts. These findings point to the involvement of structural factors in the interaction of external TPNH with the mitochondrial terminal electron transport system. It would appear from these data that the failure of PURVIS[4] to demonstrate a TPNH oxidase activity might be due to such factors rather than, as he implies, to an absence of TPNH-cytochrome *c* reductase from rat-liver mitochondria, with transhydrogenase being the only pathway for mitochondrial TPNH oxidation. This latter conclusion is also in conflict with the earlier finding of REYNAFARJE AND POTTER[11] that aging of rat-liver mitochondria for 2 h at 30° in a hypotonic medium resulted in a release of all TPNH-cytochrome *c* reductase activity into the medium at the same time as the transhydrogenase activity was recovered in the recentrifuged mitochondrial pellet.

In contrast to the TPNH oxidase activity, the transhydrogenase activity varied widely from one preparation to another. Out of ten experiments, eight gave lower transhydrogenase values than the one shown in Table I, and only in one experiment was a higher value, approaching PURVIS's figure, obtained. It should be pointed out in this connection that also in PURVIS's paper[4], a relatively low liver transhydrogenase activity, about 3 times lower than the one stated in his final scheme, can be deduced* from his experiment (Fig. 1, right) where the reduction of 2,6-dichlorophenolindophenol by isocitrate was measured in the presence of TPN and TPN + DPN. In the present experiments, the transhydrogenase activity could not be increased by increasing the concentration of TPNH or DPN, indicating that structural factors were not involved to the same extent as in the case of the TPNH oxidase. One way of explaining the observed fluctuations might be that, besides true transhydrogenase, mitochondria also contain so-called dual-specific dehydrogenases, which, depending on the presence of trace-amounts of their substrates, may transfer hydrogen from TPNH to DPN, thus adding to the total transhydrogenase activity of the system. Similar mechanisms have recently been proposed to operate in the extra-mitochondrial space of the cytoplasm, mediated by lactic dehydrogenase[13] and by certain steroid dehydrogenases[14]. It may be recalled in this connection that liver glutamic dehydrogenase, a mitochondrial enzyme[15] of potent activity[16], is known[17–19] to react with DPN and TPN at comparable rates. Investigations along these lines are now in progress.

In a recent paper, STEIN *et al.*[5] reported liver mitochondrial transhydrogenase activities considerably exceeding those obtained both in this laboratory[2–3] and by PURVIS[4]. These data are based on the use of acetylpyridine-DPN as a hydrogen acceptor. However, since this compound has a more positive potential than has DPN, the specificity and quantitative adequacy of this method may appear doubtful when applied to such a complex system as mitochondria. Acetylpyridine-DPN has recently been shown by the same authors[20] to react with the purified mitochondrial DPNH diaphorase. The reported failure[5] of the same compound to react with the purified TPNH diaphorase as well, or with TPNH in heat-treated mitochondria, does not seem conclusively to eliminate the possibility that it may react with TPNH diaphorase or TPNH-cytochrome *c* reductase in the enzymically more complex, non-heat-treated mitochondrial system. STEIN *et al.*[5] also criticize, on purely theoretical grounds, the concept previously advanced by this laboratory[12] that lactic dehydrogenase may act as a regulator of cytoplasmic TPN-linked oxidations. Since the submission of STEIN *et al.*'s paper[5], three reports[21–23] have appeared implicating an interaction between lactic dehydrogenase and TPNH (or TPN) in intact cells.

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* This deduction was made as follows: The difference in slopes between the TPN- and (TPN + DPN)-lines in PURVIS's[4] Fig. 1 (right) was estimated at 6 min to be 0.13 ΔE_{600}-units. Converted on the basis of the indicated protein content of 0.37 mg and final volume of 3 ml, and with an extinction coefficient of $21 \cdot 10^3$ l. mole^{-1} for 2,6-dichlorophenolindophenol[12], this value yields a transhydrogenase activity of $0.13 \cdot 3/(0.37 \cdot 6 \cdot 21) = 0.0084$ μequiv. 2H/min/mg protein. The corresponding value in PURVIS's[4] final scheme is 0.026.

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The assimilation and metabolism of guanidine by a strain of *Pseudomonas aeruginosa*

Although it is known that certain bacteria can desimidate substituted guanidines[1–6] and a creatinine desimidase has been partially purified[7], there has been no report on the bacterial metabolism of guanidine.

A strain of *Pseudomonas aeruginosa* was grown in 100 ml of Bacto Nutrient Broth for 24 h at 35°. After washing in water by repeated centrifugation the cells were suspended in 10 ml 0.05 M Na-K-phosphate, pH 7.6, and 0.5 ml of this used in a final volume of 2.0 ml. After incubation at 37°, 0.2 ml 20 % trichloroacetic was added and after centrifugation aliquots of the supernatant were taken for the estimation of guanidine by the method of Weber[8] using a 490 $m\mu$ filter. Methylguanidine was also estimated by this method and equivalent amounts gave 85 % of the color produced by guanidine.

When 100 μg guanidine · HCl were added to the washed cells 10–15 μg disappeared in 15 min. After that no further disappearance was evident unless an oxidizable substrate such as pyruvate was added. 30–60 min after the addition of pyruvate the rest of the guanidine began to disappear and continued to completion. This is shown in Fig. 1. This disappearance, *i.e.* assimilation, of guanidine depended on the presence of an oxidizable substrate because assimilation ceased when oxidation ceased. Thus, within limits, the amount of guanidine assimilated should be proportional to the amount of substrate added. Fig. 2 shows that this is so for pyruvate in the range of 1.0–2.5 mg. The assimilation of NH_4^+ is also dependent on oxidation and NH_4^+ completely inhibited guanidine assimilation until no extracellular NH_4^+ remained.

Following assimilation, guanidine was metabolized since none was found in the cells after they were broken by sonic vibration. The most likely mechanism is desimidation to form NH_4^+ and urea followed by the breakdown of urea by urease. Urease can be induced in these cells by the addition of pyruvate[9]. (Endogenous purines are metabolized to urea in the presence of an oxidizable substrate.) Thus, if urea is formed from guanidine, the presence of previously induced urease should increase the rate of guanidine assimilation. Fig. 3 shows that a 60-min pre-incubation of the cells with pyruvate before the addition of guanidine does this. Pre-incubation with pyruvate has no effect on NH_4^+ assimilation.

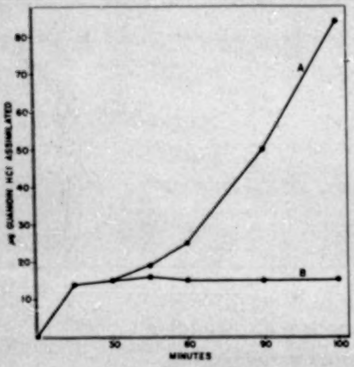

Fig. 1.

Fig. 1. The assimilation of 100 µg guanidine·HCl in the presence (A) and absence (B) of 2.0 mg Na pyruvate.

Fig. 2. The assimilation of 100 µg guanidine·HCl after 240 min incubation in the presence of differents amounts of Na pyruvate.

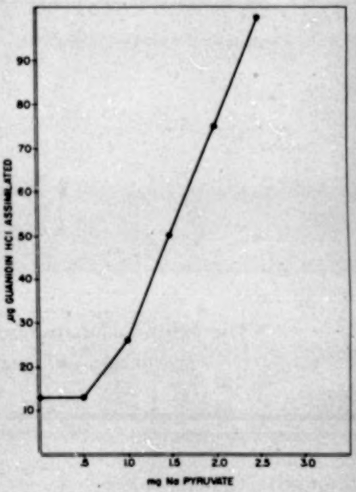

Fig. 2.

It was possible to show that all the nitrogen in guanidine was available for utilization by the cell. This was done by inducing the enzymes for the oxidation of benzoate and comparing the effect of guanidine with that of an equivalent amount of nitrogen added as $(NH_4)_2SO_4$. Shortly after the oxidation of benzoate began, energy was available for the assimilation of the NH_4^+, and the apparent oxidation rate of benzoate and its products was increased. This is shown in Fig. 4 (non-nitrogenous compounds in equimolar amounts were without effect on the oxidation rate). The curves on the left represent the increase in oxidation rate in the presence of 4.2 and 8.4 µg $(NH_4)_2SO_4$ over that of the control. When guanidine was substituted for $(NH_4)_2SO_4$ the increase began later and proceeded at a slow rate at first. With urea the increase began still later. But once urease was induced, the oxidation rate in the presence of both guanidine and urea increased rapidly. It is possible to infer from this that the imino nitrogen of guanidine became available first, followed by the other two nitrogen atoms once urease was induced. The extent of the increase in oxidation rate was roughly proportional to the amount of nitrogen present as shown for $(NH_4)_2SO_4$ in Fig. 4. The proportionality was also true for the other compounds. The increase in rate ceased only because the nitrogen was limiting.

It seems probable that the latent period in the assimilation of guanidine represents the time necessary for the induction of a desimidase. Two facts indicate that this is so. (a), Chlortetracycline (0.05 μg/ml), which inhibits enzyme induction in this organism, completely inhibited guanidine assimilation when added at the beginning but caused only partial inhibition when added during the linear phase of the reaction.

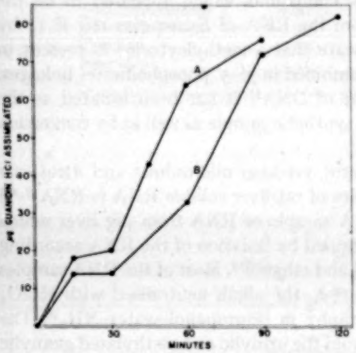

Fig. 3. The effect of 60 min pre-incubation with pyruvate on the assimilation of 100 μg guanidine·HCl. A, pre-incubated. B, control.

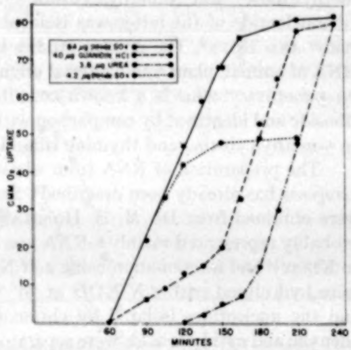

Fig. 4. The increase in oxidation rate of 0.5 mg Na benzoate in the presence of various nitrogenous compounds. The oxygen uptake of benzoate alone has been subtracted.

(b) Increasing the nitrogen pool by allowing the cells to assimilate NH_4^+ before the addition of guanidine increased the rate of guanidine assimilation (NH_4^+ completely inhibits urease induction).

All the above facts apply to methylguanidine except that there was a longer latent period before assimilation began. Since all three of its nitrogen atoms were available to the cell and since urease does not hydrolyze methylurea, methylguanidine was probably demethylated. Creatinine was assimilated after a much longer latent period and apparently only one of its nitrogen atoms became available. There was no evidence that glycocyamine was metabolized.

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The isolation of 5-methylcytidine from RNA

Studies in recent years have indicated the presence in RNA from a number of sources of several minor components in addition to the four main nucleotides. These include pseudouridine[1–4], thymine[5], methylated adenines[5,6], methylated guanines[6,7], 2-methylribose[8] and 5-methylcytosine[9,10]. A compound which appeared to be the ribonucleotide of the latter was isolated from the RNA of *Escherichia coli* K 12 by AMOS AND KORN[9]. The present studies indicate that 5-methylcytosine is present in RNA of animal, plant and bacterial origin combined in 3′–5′ phosphodiester linkages. As 5-methylcytosine is a known constituent of DNA[11] it has been isolated as the riboside and identified by comparison with a synthetic sample as well as by conversion to 5-methylcytosine and thymine riboside.

The preparation of RNA from wheat germ, rat-liver microsomes and *Aerobacter aerogenes* has already been described[5]. Samples of rat-liver soluble RNA (s-RNA[12,13]) were obtained from Dr. M. B. HOAGLAND. A sample of RNA from pig liver which probably represented mainly s-RNA was obtained by isolation of the RNA according to KIRBY[14] and fractionation using 2 *M* NaCl and ethanol[15]. Most of the RNA samples were hydrolysed with 1 *N* KOH at 30° for 18 h, the alkali neutralised with $HClO_4$ and the nucleotides isolated by chromatography in isopropanol–water–NH_3[16]. The adenylic and cytidylic acids were separated from the uridylic and methylated guanylic acids by electrophoresis at pH 2.6[7] and rechromatographed in isopropanol–water–NH_3 to remove phosphate. The nucleotides were treated with prostatic phosphomonoesterase[17] under the conditions previously used[5], and the resulting nucleosides were separated by chromatography in isopropanol–water–HCl[11]. The 5-methylcytidine was separated from the cytidine by chromatography of the leading half of the cytidine band in *n*-butanol–water–NH_3[18] for 36–72 h. Double spotting of the cytidine and 5-methylcytidine occurred in the butanol solvent but could be prevented if the material eluted from the HCl chromatogram was first chromatographed in isopropanol–water–NH_3. It was found that satisfactory results could be obtained if the chromatogram was run for 3–6 h in the isopropanol–water–NH_3, then dried and the same paper chromatographed in the *n*-butanol.

As in previous studies[5,7] the riboside was identified by comparison with a known sample of 5-methylcytidine. Attempts to synthesize this riboside using inosine, 5-methylcytosine and the nucleoside phosphorylase previously used[5,7] were unsuccessful but I was fortunately able to obtain a sample of the ribofuranoside recently synthesized by FOX *et al.*[19]. In four solvent systems the nucleoside from RNA had the same R_F as the synthetic riboside (Table I) and its spectra at pH 1 and 13 corresponded closely to those obtained by FOX *et al.* at pH 0 and 14 respectively (Fig. 1). On heating my nucleoside with 72% $HClO_4$ for 2 h at 100° I obtained a compound identified chromatographically in four solvents (Table I) and spectroscopically as 5-methylcytosine. AMOS AND KORN[9] reported that under similar conditions their nucleotide was converted to thymine, but no detectable amount of this compound was formed in my experiments. As expected on treatment with HNO_2 the nucleoside was converted to a compound identified chromatographically and spectroscopically as thymine riboside[5].

Abbreviations: RNA, ribonucleic acid; s-RNA, soluble RNA; DNA, deoxyribonucleic acid.

TABLE I

R_F VALUES OF 5-METHYLCYTOSINE, 5-METHYLCYTIDINE AND OTHER RIBOSIDES IN FOUR SOLVENT SYSTEMS

	isopropanol –water–HCl	*isopropanol –water–NH_3*	*n-butanol –water–HCOOH*[18]	*n-butanol –water–NH_3*
5-Methylcytosine	0.60	0.59	0.25	0.30
5-Methylcytidine	0.57	0.57	0.12	0.12
Cytidine	0.55	0.54	0.10	0.10
Adenosine	0.41	0.59	0.17	0.18

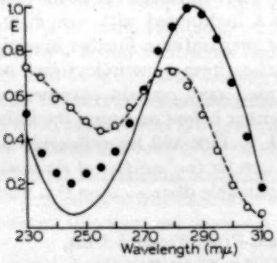

Fig. 1. U.v.-absorption spectra of 5-methylcytidine from Fox *et al.* at pH 0 (———) and at pH 14 (— — —). Points are for a sample isolated from rat-liver soluble RNA at pH 1 (●) and pH 13 (○). Spectra in acid and alkali are for solutions of the same concentration, the spectrum in acid being plotted to give a value for E of 1.0 at 287 mμ.

The R_F values of the nucleoside in the butanol solvents agreed with the presence of ribose rather than deoxyribose as the deoxyriboside would have a higher R_F in these solvents[20]. The presence of ribose was also indicated by the formation of a negatively-charged borate complex: both the synthetic and the nucleoside from RNA moving with cytidine on paper electrophoresis in borate, pH 9.2. In addition, 5-methylcytosine was identified as the major u.v.-absorbing product when the nucleoside was treated successively with periodate and alkali[21, 5].

5-Methylcytidine was also isolated from a sample of pig-liver RNA which was hydrolysed with snake venom. The RNA (4 mg) was incubated at 37° in glycine buffer, pH 9, with 1 mg Russell viper venom for 6 h, chloroform being added to prevent bacterial contamination. The products were chromatographed in isopropanol–water–NH_3 and the band containing adenosine, cytidine and uridine was subjected to paper electrophoresis in phosphate, pH 2.6. The fastest moving band of cytidine and adenosine was chromatographed in isopropanol–water–HCl and the 5-methylcytidine isolated as described above. It was identified by its R_F values in the four solvents, its spectra and by conversion to 5-methylcytosine. As hydrolysis with snake venom would involve formation of the 5′ nucleotides[22] while alkali cleaves RNA to the 2′ and 3′ phosphates[23] it seems that the 5-methylcytidine residues must be present in the polynucleotides mainly in 3′–5′ phosphodiester linkages.

The 5-methylcytidine was estimated spectrophotometrically using a value of ε of $12.5 \cdot 10^3$ at 287 mμ in 0.1 *N* HCl[19]. As observed with the other additional components[24] the highest proportion was detected in rat-liver s-RNA (10 moles/100 moles uridine) and a similar proportion (6 moles/100 moles) was found in the pig-liver RNA. A smaller proportion was found in rat-liver microsome RNA (0.4 mole/100 moles uridine) and even smaller proportions in RNA from wheat germ (0.2 mole/100 moles uridine) and *A. aerogenes* (0.15 mole/100 moles uridine).

It has been suggested[9] that the thymine we previously detected in RNA[5] arose from 5-methylcytidine residues during the alkaline hydrolysis. The detection of 5-methylcytosine but not thymine[5,10] in both the rat-liver fractions after alkaline hydrolysis seems to rule out this possibility. It is noticeable, however, that the sources such as wheat germ and *A. aerogenes*, which contain comparatively high proportions of thymine, contain little 5-methylcytosine. A small proportion of thymine was detected in the pig-liver RNA hydrolysed with venom or alkali and the proportion seemed variable in different preparations. Further investigations will be necessary to establish if the thymine arises from 5-methylcytosine residues, but it seems quite possible that the tissues used may contain enzymes capable of deaminating the 5-methylcytidine residues either before or during the isolation of the RNA.

I wish to thank Dr. J. J. Fox and his colleagues for the sample of 5-methylcytidine, Dr. M. B. Hoagland for the samples of rat-liver RNA and I am grateful to Dr. J. D. Smith for much valuable discussion on this work.

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The application of the CERIOTTI reaction to determination of deoxyribonucleotides

CERIOTTI has described the colorimetric determination of deoxyribonucleic acid by a reaction with indole in 3 *N* HCl[1,2]. The earlier method of DISCHE using a lower concentration of HCl is less sensitive[3]. However, of the monodeoxyribonucleotides (or deoxyribosides), only thymidylic acid[4] has been reported to yield a color in the CERIOTTI procedure. A colorimetric response of several additional deoxyribonucleotides is described herein.

The procedure used is similar to that described by CERIOTTI[1]. To 2 ml of sample containing 0.100 µmole of deoxyribosidic compound in a 16 × 125 mm culture tube is added 1 ml 0.04 % indole and 1 ml conc. HCl (sp.gr., 1.18) with mixing after each addition. The tube is closed with a Teflon-lined screw-cap and placed into a boiling water bath within 5 min after acid addition. After heating for a given time, the tubes are cooled in a water bath at 20°. Absorbancies are determined at 490 mµ in curvettes with a 1-cm light path using a Beckman model DU spectrophotometer. Unless otherwise stated, absorbancies are without chloroform extraction. Chloroform extraction, when necessary, is carried out prior to rereading absorbancies, by twice shaking with 4 ml of reagent-grade chloroform (Mallincrodt Chemical Works, St. Louis, Missouri) and aspirating off the chloroform. Readings are against a reagent blank treated like the sample.

Fig. 1 shows the absorbancy at 490 mµ, and the effect of heating time for 5 deoxyribosidic compounds after being subjected to the indole–HCl treatment. The color development from each compound indicates the color response is general for deoxyribonucleotides. This is in contradiction to the inference of CERIOTTI that probably only the purine deoxyribonucleotides should respond[1]. However, the absorbancy arising from deoxycytidylic acid and deoxyuridine is less for a given concentration, than for the two purine deoxyribonucleotides and thymidylic acid. The increase in colorimetric response of the pyrimidine deoxyribotide compounds on heating beyond 10 min is in distinct contrast to the decrease seen with the purine

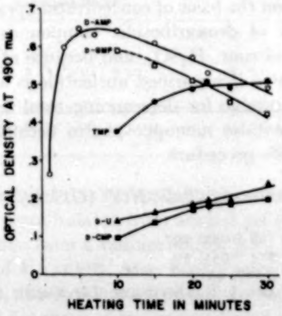

Fig. 1. Color formed by deoxyribosidic compounds on heating with indole and HCl for various times. 0.10 µmole in 4 ml final volume. ○—○ Deoxyadenylic acid (D-AMP); □—□ Deoxyguanylic acid (D-GMP); ●—● Thymidylic acid (TMP); ▲—▲ Deoxyuridine (D-U); ■—■ Deoxycytidylic acid (D-CMP).

deoxyribonucleotides. Heating beyond 30 min gives poor results because of the appearance of turbidity. The absorbancy at 490 mμ resulting from heating deoxyribosidic compounds with indole and HCl for 22 min is presented in Table I. The results show that the method is not completely specific for deoxyribosidic compounds, but that absorbancy arising from a ribosidic compound is readily extracted by chloroform, as contrasted to the poor extraction from a deoxyribosidic compound. Hence, specificity may be checked by an absorbancy reading after chloroform extraction. Duplicate determinations of deoxyribosidic solutions varied by an average of 2 % from the mean and determinations of different days varied in a range of 10 %. The reagent blank for 22-min heating has an absorbancy of around 0.042 against water. The determination may not be carried out in the presence of ammonium formate since upon heating 0.5 *M* ammonium formate containing indole and HCl, a dense orange color appears which is removed only by repeated extractions into chloroform, in which it assumes a pink color.

TABLE I

RESULT OF HEATING VARIOUS COMPOUNDS WITH INDOLE AND HCL FOR 22 MIN

Compound (0.10 μmole in 4 ml final sol.)	*Absorbancy at 490 mμ*	*after two extractions with $CHCl_3$*
Deoxyadenylic acid	0.528	0.468
Deoxyguanylic acid	0.511	0.450
Thymidylic acid	0.503	0.444
Deoxycytidylic acid	0.164	0.166
Deoxyuridine	0.196	0.148
Adenylic acid	0.085	0.014
Guanylic acid	0.108	0.028
Cytidylic acid	0.005	
Uridylic acid	0.010	

A method previously described for purine and pyrimidine deoxyribonucleotides involves reaction with cysteine and conc. H_2SO_4[5,6]. The sensitivity of this method for deoxycytidylic acid approximates that of the indole–HCl method in terms of concentration in the final reaction volume; however, the indole–HCl method is three or more times as sensitive on the basis of concentration prior to reagent addition. The indole–HCl determination of deoxyribosidic compounds described here is useful because it avoids the use of conc. H_2SO_4, and because of its directness and ease. It may be applied to determine if a purified nucleotide is a deoxyribonucleotide. The method is not generally suitable for determining total deoxyribosidic content of a mixture of deoxyribonucleotides monophosphates because of their differing molar extinction coefficients in this procedure.

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Changes in the sodium and potassium contents of cell nuclei after irradiation

The ability of thymus nuclei to retain sodium and potassium during isolation in aqueous media has been studied by ITOH AND SCHWARTZ[1] who concluded that sodium was the more firmly bound of the two elements. A connection between the release of nucleotides and potassium from isolated calf-thymus nuclei, and loss of the ability to perform nuclear phosphorylation was reported by OSAWA, ALLFREY AND MIRSKY[2]. In view of the marked radiosensitivity of nuclear phosphorylation[3,4], it seemed desirable to see whether radiation could also bring about this loss of nuclear potassium. In this connection it is of interest that changes have been described in the plasma and urinary electrolyte levels of irradiated animals[5,6], and a loss of potassium found to occur from the cells of the rat spleen 2 h after 1000 R of X-rays[7].

Rats of 80–120 g in weight were exposed to total-body X-radiation. The animals were killed by decapitation 1 h later, the spleen and thymus glands removed and nuclei prepared from them in buffered 0.25 *M* sucrose–0.0033 *M* $CaCl_2$ as described previously[4] except that the final centrifuging and resuspension were performed in medium containing no buffer. This was done to ensure a low sodium background. The sodium and potassium contents of nuclear preparations from the spleen and thymus glands were then determined by flame photometry of solutions of the residues of tissues which had been heated for 4 h with HNO_3. An oxy-acetylene flame was used, and corrections applied for the mutual interference of the sodium and potassium emissions[8]. DNA was estimated by the method of BURTON[9] and expressed as DNA phosphorus. It was used as a reference standard. It can be seen in Table I that there is a loss of both sodium and potassium from the nuclei of the irradiated tissues, but the control values themselves are rather variable.

TABLE I

CHANGES IN THE SODIUM AND POTASSIUM CONTENTS OF NUCLEI ISOLATED FROM TISSUES OF RATS EXPOSED TO TOTAL-BODY X-RADIATION 1 h PREVIOUSLY

Tissue	*Element*	*Control content µequiv./mg DNAP*	*Content of irradiated nuclei % control level*		
		Dose:	*25 R*	*50 R*	*1000 R*
Thymus	Potassium	5.55; 1.50; 4.16; 5.73	13, 21	27	34
	Sodium	3.90; 5.47; 2.04; 6.12.	56, 71	45	15
Spleen	Potassium	14.2; 5.17; 2.88; 6.52; 7.16	63, 69	113, 42	27
	Sodium	11.0; 8.77; 7.96	43.	33	17

To overcome the difficulty of this variation in the control values, we carried out irradiations *in vitro*. Nuclei were isolated from normal rat spleen, and portions of the suspensions exposed to γ-rays from a radium source (two 250 mC sources, dose rate calibrated by the method of MILLER[10]). After centrifuging these samples (completed within 5 min of irradiation), the sodium and potassium contents of the supernatants were determined, corrected for those of the un-irradiated suspensions and expressed as percentages of the total content of each nuclear preparation. These results appear in

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Abbreviations: DNA, deoxyribonucleic acid.

Figs. 1 and 2 and show that loss of potassium from spleen nuclei occurs less readily than that of sodium, but that both elements are largely removed by doses of radiation of the same order as inhibit nuclear phosphorylation[4].

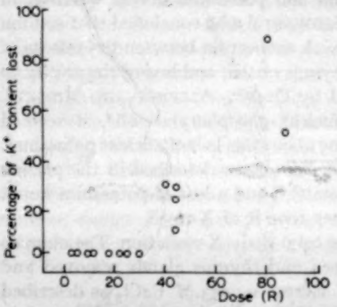

Fig. 1. The loss of potassium from isolated rat spleen nuclei as a function of the dose of γ-rays received. Losses are calculated as percentage of the total nuclear content lost into the supernatant 5 min after irradiation.

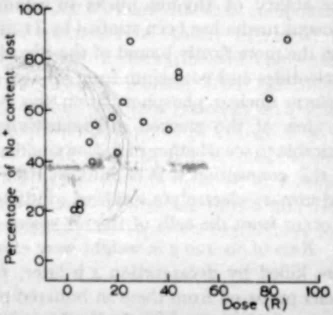

Fig. 2. The loss of sodium from isolated rat spleen nuclei as a function of the dose of γ-rays received. Losses are calculated as percentage of the total nuclear content lost into the supernatant 5 min after irradiation.

These observations suggest that the mechanism of the radiation lesion in nuclear phosphorylation involves the loss of bound sodium and potassium. The difference between the properties of nuclear and mitochondrial oxidative phosphorylation is emphasized by the fact that although the latter process is also dependent upon bound potassium[11], it is known to be very resistant to radiation *in vitro*[12].

I wish to express my thanks to Dr. L. A. Stocken for his interest and helpful suggestions during the course of this work; to Mr. M. J. Corp of the M.R.C. Radiobiological Research Unit, Harwell, for irradiating the animals and to Mr. R. Hems for advice on flame photometry. I am also grateful to the Medical Research Council for a Training Scholarship and to the Rockefeller Foundation for grants towards the cost of apparatus.

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Inactivation of follicle-stimulating hormone by enzymic release of sialic acid

In 1948 it was observed by WHITTEN[1] that the biological activity of crude chorionic and pituitary gonadotropins was lost when the hormone materials, prior to injection into animals, were treated with influenza virus or with the receptor-destroying enzyme (RDE) of *Vibrio cholerae*. Over the last years the influenza-virus enzyme and RDE have been recognized[2] as neuraminidases and their action defined[3] as "cleaving the α-ketosidic linkage joining the terminal acylated neuraminic acid to another sugar or sugar derivative". Recently BROSSMER AND WOLTER[4] confirmed WHITTEN's observation and reported that the loss of biological activity of chorionic gonadotropin on treatment with the above catalytic agents was accompanied by the release of N-acetylneuraminic acid, as shown by paper chromatography. Since their work was carried out on a qualitative basis only and since the influenza virus and RDE used were crude preparations, it seemed desirable to investigate quantitatively the effect of highly purified α-neuraminidase on the chemical composition and biological activity of a standard preparation of FSH.

25 mg of the ovine FSH preparation* was dissolved in 4.0 ml water. 3 ml of the solution were treated with highly purified RDE** (50,000 units, 35 μg protein) at pH 6.0 and in the presence of 0.005 M Ca^{++} at 35° for 18 h. The control (residual 1.0 ml of solution) was kept under the same conditions but without RDE. Control and assay were then quantitatively transferred to dialysing tubings and dialysed against 10 vol. water at 0° for 48 h. The dialysates were lyophilized. Sialic acid determinations[5] on aliquots of the contents of the dialysing bags and on aliquots of the lyophilized materials gave the results shown in Table I.

TABLE I

RELEASE OF SIALIC ACID BY NEURAMINIDASE FROM FSH

	Dialysed material		*Dialysate*	
	FSH	*FSH-RDE*	*FSH*	*FSH-RDE*
Sialic acid content*	5.0	0	0	5.15

* mg sialic acid (expressed as N-acetylneuraminic acid) obtained/100 mg FSH.

The enzymically released compound was identified as sialic acid by conversion to pyrrole-2-carboxylic acid (0.1 N NaOH, 100°, 20 min) using the isolation procedure described previously[6]. The isolated compound (solvent, ethanol) had a u.v. absorption maximum at 263 mμ identical with that of authentic pyrrole-2-carboxylic acid. The identity of the two compounds was confirmed by paper chromatography (*n*-butanol–pyridine–water (6:4:3, v/v)).

The gonadotropic activity of the control and assay (RDE-treated, dialysed FSH) was examined in 3-week-old female mice. A group of 6 animals was injected with one

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Abbreviations: FSH, follicle-stimulating hormone; RDE, receptor-destroying enzyme.

* The FSH preparation, code name NIH-FSH-S-1, was a gift from the National Institutes of Health, Bethesda, U.S.A.

** Kindly prepared and presented by Drs. G. ADA and E. FRENCH, Melbourne.

of five graded doses of either control (0.001–0.1 mg FSH) or assay (0.01–0.9 mg); the mice also received 40 I.U. of chorionic gonadotropin[7]. After appropriate time the ovarian and uterine weights were recorded. Typical dose-response regressions were obtained with 0.03 mg and more FSH, whereas the highest dose (0.9 mg) of the RDE-treated FSH used did not cause any significant increase in ovarian and uterine weights. Similar results were obtained with rats (without simultaneous application of chorionic gonadotropin).

The following conclusions may be drawn from the experimental results. (a) All sialic acid residues in FSH are terminal, linked α-ketosidically to their partner and are accessible to α-neuraminidase. (b) Enzymic release of the sialic acid residues reduces the biological activity of FSH by 97 % or more. A more exact estimate of the residual biological activity, if any, would have required amounts of pure FSH not available at present. The data presented taken together with the well known capacity of certain mucoproteins to inhibit in high dilution haemagglutination by influenza viruses, a capacity lost on treatment with α-neuraminidase, are some indication of the range of biological activities imparted to mucoproteins by the presence in them of terminal sialic acid residues.

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Metabolism of [4-^{14}C]corticosterone by fibroblasts, strain U12-79

The physiological importance of the fibroblast and its ability to resist destruction in the presence of corticosteroids in an inflammatory process has been shown by DOUGHERTY *et al.*[1,2]. It has been demonstrated that the ability of corticosteroids to inhibit inflammation is a function of structure and that compounds which have a 17α-OH group are the most potent anti-inflammatory steroids[3]. The metabolism of various steroids by connective tissue[4] and fibroblasts propagated *in vitro*[5,6] have been studied in these laboratories. The data to be presented in this report indicate that a permanent strain of fibroblasts converts corticosterone to $11\beta,20\beta,21$-trihydroxypreg-4-en-3one.

Strain U12–79[7,8] was propagated in solution S103[9] fortified with 5 % dialysed

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horse serum (medium 79) according to method described previously[7,8]. In expt. 1, 10 ml of medium 79 supplemented with 0.3 μg/ml [4-^{14}C]corticosterone (specific activity, 4.2 μC/mg) was added to each of 5 T60 flasks containing a total of $3.4 \cdot 10^7$ cells. Expt. 2 was identical with 1 except that 0.05 μg/ml unlabeled cortisol was added to the 79 in addition to [4-^{14}C]corticosterone. After 72 h at 37° the cells and medium in each experiment were combined and stored at —20° until analysed. Aliquots of each medium incubated with [4-^{14}C]corticosterone for 72 h at 37° in the absence of cells served as controls. Each reaction mixture was extracted with acetone–chloroform[4]. This fraction was chromatographed according to the methods of ZAFFARONI[10]. Isotope techniques for the identification and quantitation of steroids were used as described previously[11, 12].

The isotope data indicated that at least 90 % of the steroid was recovered in the chloroform fraction from each of the experimental and control incubation mixtures. In Expts. 1 and 2 only one radioactive peak other than that of the un-metabolized corticosterone was observed when the extracts were chromatographed in the chloroform–formamide system[10]. In the control incubations, only [4-^{14}C]corticosterone was observed upon chromatography. This compound was checked by chromatography in chloroform–formamide (R_F, 0.7) and in benzene–formamide (R_F, 0.1). The acetate derivative was prepared[11], and it migrated at the same rate as authentic non-radioactive corticosterone acetate in benzene–formamide (R_F, 0.5).

The unknown metabolite migrated at a rate (R_F, 0.29) in chloroform–formamide which is slower than cortisol and faster than substance "E" of REICHSTEIN (11β, 17α,20β,21-tetrahydroxypregn-4-en-3-one). These results suggested that the compound could be either 11β,20β,21-trihydroxypregn-4-en-3-one or 20 epi substance "T" of REICHSTEIN (20α,21-dihydroxypregn-4-ene-3,11-dione.)

Unlabeled 11β,20β,21-trihydroxypregn-4-en-3-one was added to an aliquot of radioactive unknown and the mixture was chromatographed in chloroform–formamide. Both compounds migrated at the same rate. The mixture was then acetylated and chromatographed in benzene–formamide. Again the radioactivity migrated at the same rate, indicating the formation of 11β-hydroxy-20β,21-diacetoxypregn-4-en-3-one. This mixture was further oxidized with CrO_3[11] and chromatographed in the benzene–formamide system. The polarity of both the radioactive unknown and authentic 11β,20β,21-trihydroxypregn-4-en-3-one diminished, without separating and forming 20β,21-diacetoxypregn-4-ene-3,11-dione as final product. The foregoing data indicate that corticosterone is converted to 11β,20β,21-trihydroxypreg-4-en-3-one by the U12-79 strain of fibroblasts. There was a 9.8 % and 8.0 % conversion in Expts. 1 and 2 respectively.

Authentic, unlabeled 11β-hydroxyandrost-4-ene-3,17-dione, 20 epi substance "T" of REICHSTEIN and cortisol were added as carriers to the initial chloroform extract in all cases. Upon chromatography and formation of derivatives, no radioactivity was observed in the same zone of the chromatogram where the carrier was present.

The U12-79 strain of fibroblasts[5], loose connective tissue[4], and osteosarcoma cells from beagle dogs [3] convert cortisol to corticosterone. In earlier studies with U12-79[5] employing cortisol as substrate, 11β,20β,21-trihydroxypregn-4-en-3-one did not accumulate in sufficient quantities to be identified. The data obtained in Expt. 2 indicate that corticosterone is not converted to cortisol by U12-79. It appears that a similar situation may exist *in vivo* since the antiphlogistic activity of corticosterone is much less than that of cortisol. Similarly, compounds reduced in the 20 position lose their antiphlogistic activity[14]. It is of interest in this connection, that most of the products of cortisol and progesterone metabolism by U12-79 are much less inhibitory to the growth of this strain than are the corresponding hormones[15]. It would therefore appear that both cells *in vivo* and those propagated *in vitro* convert the active hormone to less active products. The relationships between these transformations and the physiological effects of the corticosteroids on fibroblasts[3] remain to be elucidated.

The results obtained with corticosterone are in agreement with earlier observations[5, 6, 16, 17] which indicate that the steroid nucleus is not metabolized. Similarly the data are in agreement with other observations which indicate that neither cells propagated *in vitro* nor extra hepatic tissues can actively reduce the 3 position of the steroid molecule. This reaction and conjugation of steroids appear to be carried out mainly by the liver[18, 19].

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Preliminary Notes

Function of vitamin B_{12} in methylmalonate metabolism

I. Effect of a cofactor form of B_{12} on the activity of methylmalonyl-CoA isomerase

The study of BARKER *et al.*[1] showing that a derivative of pseudo-vitamin B_{12} functions in the reversible conversion of glutamate to β-methylasparate in *Clostridium tetanomorphum* suggested to us the possibility of a similar involvement of vitamin B_{12} in the isomerization of methylmalonyl-CoA to succinyl-CoA in the propionate metabolism pathway[2] in the animal.

Studies carried out using enzyme preparations and incubations essentially according to the methods of FLAVIN AND OCHOA[2] for the incorporation of $^{14}CO_2$ into succinate with propionyl-CoA used as substrate showed a several-fold reduction in vitamin B_{12}-deficient rats[3]. The difference in incorporation between the B_{12}-normal and the deficient rats was primarily due to a reduction in the isomerase step, since methylmalonate formation was essentially the same for the deficient and normal groups. This is in agreement with similar results obtained by SMITH AND MONTY[4].

Attempts to restore the activity of the isomerase from B_{12}-deficient animals by

TABLE I

INCORPORATION OF $^{14}CO_2$ INTO METHYLMALONIC, SUCCINIC, AND OTHER ACIDS FORMED FROM PROPIONYL-CoA BY MITOCHONDRIAL PREPARATION FROM THE LIVERS OF VITAMIN B_{12}-NORMAL AND DEFICIENT RATS

System	*Total incorporation**		*Incorporation in succinate**	
	Nutritional status of rats		*Nutritional status of rats*	
	$-B_{12}$	$+B_{12}$	$-B_{12}$	$+B_{12}$
Expt. I				
Complete system (CS.)	1312	1446	81	1236
CS — propionyl CoA	9	7	1	0
CS + B_{12} (100 mμg)	1292	1272	82	763
CS + 2 μmoles CN	1377	1451	92	1106
CS + DMBC (10 mμg)	1195	1374	208	1167
CS + DMBC (50 mμg)	1209	1289	308	1101
CS + DMBC (100 mμg)	1253	1336	599	1121
Expt. II				
Complete system	1160	1043	96	757
CS + B_{12} (100 mμg)	1025	1154	64	773
CS + 2 μmoles CN	1042	1207	64	858
CS + DMBC (100 mμg)	1108	1191	747	1087
CS + DMBC (100 mμg)	1103	1151	807	881
CS + DMBC (400 mμg)	1156	1116	907	871

* In counts/min/mg protein added. Total incorporation includes activity present in methylmalonate, succinate, malate and fumarate.

Abbreviations: B_{12}, vitamin B_{12}; DMBC, 5,6-dimethylbenzimidazole-B_{12} coenzyme; Tris, tris(hydroxymethyl)aminomethane; GSH, glutathione; CoA, coenzyme A; ATP, adenosine triphosphate.

addition of vitamin B$_{12}$ *in vitro* were unsuccessful, as were attempts to inhibit with cyanide; however, when 5,6-dimethybenzimidazole-B$_{12}$ coenzyme[5] was used the isomerase activity was completely restored to normal (Table I).

Two experiments were carried out, each in duplicate, involving two normal and two deficient rats. The reaction mixture complete system contained Tris buffer, pH 7.4, 400 µmoles; MgCl$_2$, 10 µmoles; GSH, 10 µmoles; ATP, 10 µmoles; propionyl-CoA[6], 1 µmole; NaH^{14}CO$_3$, 10 µmoles (10^6 counts/min); and buffer extract of acetone-dried rat-liver mitochondria[7,8], containing 18–25 mg protein. The final volume was 1.7 ml. The mixtures were incubated at 28° in an atmosphere of $O_2 + CO_2$ for 1 h and the reaction was stopped by the addition of 0.3 ml 2 *N* HClO$_4$. Direct scintillation counting of acid-deproteinized aliquots gave the total radioactivity incorporated while counting after heating aliquots for 10 min at 100° with a few drops of 3% KMnO$_4$, and removal of excess permanganate by reduction with methanol gave the radioactivity due to succinate, since succinate is stable to this permanganate oxidation, while methylmalonate, as well as malate and fumarate, is oxidized to volatile products. The precipitated MnO$_2$ was removed by centrifugation before counting of the filtrates. All results are expressed in counts/min/mg protein (enzyme preparation) added.

The data given in Table I clearly indicate that vitamin B$_{12}$ coenzyme is a cofactor for the isomerization of methylmalonyl-CoA to succinyl-CoA.

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Nitrogen fixation in cell-free extracts of *Clostridium pasteurianum*

Biological nitrogen fixation is of fundamental importance to the existence of all living things, yet there has been little progress in understanding the chemistry of the process. Investigations at the enzyme level to define the mechanism have hitherto been blocked by failure to find adequate ways for extracting the enzymes in active

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condition. We wish now to report the discovery of reproducible procedures for extracting the nitrogen-fixing enzyme system from *Clostridium pasteurianum*.

Tracer experiments with $^{15}N_2$ and resting cells of *C. pasteurianum* revealed that fixation by intact cells was detectable only in the presence of a metabolic energy source, preferably sodium pyruvate. This information was applied in an investigation of cell-breaking techniques, and two methods were found giving active extracts. In the first method, concentrated suspensions of cells[1] in 0.05 *M* phosphate buffer, pH 6.8, were disrupted in a Hughes press[2] at — 15 to — 35° and transferred while still frozen to assay vessels or centrifuge tubes. In the second and more convenient method, dried cells were autolyzed by shaking them anaerobically in the buffer at 30° and centrifuging to remove debris. The dried cells were prepared by rotating concentrated cell pastes in a "Rinco" evaporator in a water bath at 50° under vacuum. Rigorous control of conditions in these procedures is essential, particularly since the extracts are inactivated by air.

Assays were made by adding 100 mg sodium pyruvate to 3–4 ml of the extracts in a total volume of 8 ml and exposing to $^{15}N_2$ on a shaker at 30°[3]. In 1 h under 60 atom % excess $^{15}N_2$, enrichments ranging from 0.1 to 0.6 atom % excess ^{15}N have been obtained. Activity has persisted for as long as 2 h. That these preparations are free of intact cells has been demonstrated by microscopic examination and ultracentrifugation.

Sodium pyruvate has been the most active substance found for causing fixation in these extracts. Activity could also be detected, though in lesser amounts, with α-ketobutyric acid but not with many other hydrogen donors tested including H_2. Extracts of poor activity could sometimes be improved with supplementary coenzyme A.

The enzyme system appears to be water-soluble since activity was not sedimented by centrifugation at 144,000 × *g* for 2 h. The centrifuged extracts were clear brown solutions that fixed up to 12 μg N/mg protein nitrogen initially present.

The fixation reaction seems to comprise NH_3 synthesis[4] since the $^{15}N_2$ taken up by the extracts could be recovered quantitatively as $^{15}NH_3$. The extracts show a spectral response to nitrogen. The difference spectrum under N_2 against H_2 or A shows absorption in the 300–365 mμ region distinct from oxygen-induced differences which are found at 385–420 mμ.

The techniques applicable to *C. pasteurianum* have not yet been successful for *Azotobacter vinelandii* or *Nostoc muscorum*.

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Conversion of pregnenolone to dehydroepiandrosterone

Pregnenolone (3β-hydroxypregn-5-en-20-one) as well as cholesterol have been demonstrated, in this report, to be precursors of dehydroepiandrosterone. This was accomplished by incubating [^{3}H]pregnenolone and [4-^{14}C]cholesterol with a homogenate prepared from a human adrenal-cortical adenoma* (Cushing's syndrome), adding dehydroepiandrosterone and isolating [^{3}H,^{14}C]dehydroepiandrosterone which, after chromatography, crystallization, acetate formation, and recrystallization, was shown to be radiochemically pure.

The adrenal tumor tissue (7.5 g) was homogenized in 15 ml phosphate buffer, pH 7.4, containing DPN ($5\cdot10^{-4}$ *M*) and the following constituents at $5\cdot10^{-3}$ *M*: ATP, nicotinamide, $MgSO_4$ and sodium fumarate. In addition, 40 I.U. ACTH were added together with 0.5 mg [4-^{14}C]cholesterol ($2.2\cdot10^6$ counts/min/mg) and 5.0 mg [^{3}H]pregnenolone ($1.9\cdot10^6$ counts/min/mg). The mixture was incubated in air for 3 h at 37°, then precipitated with acetone and the insoluble material extracted successively with acetone, ethyl acetate, and chloroform. The acetone fractions together with the ethyl acetate and chloroform extracts were concentrated to dryness *in vacuo* and the dry residue partitioned between heptane and 90% methanol. The methanol fraction was subjected to silica gel column adsorption chromatography and paper chromatography in a toluene-propylene glycol system and 3 mg of non-radioactive dehydroepiandrosterone were added to the material eluted from the dehydroepiandrosterone zone. This material was rechromatographed successively on paper in the heptane-propylene, cyclohexane-formamide, and the Bush B systems. The material eluted from the dehydroepiandrosterone zone had specific activities of 110 and 1450 counts/min/mg respectively for ^{14}C and ^{3}H. The compound was recrystallized three times, the acetate formed in the conventional manner and recrystallized twice. As indicated in Table I, no significant changes were detected in the specific activities of the compound with respect to the free compound or the acetate.

TABLE I

THE SPECIFIC ACTIVITY OF DEHYDROEPIANDROSTERONE AT SUCCESSIVE STAGES OF PURIFICATION

Procedure	*Specific Activity Counts min/mg*	
	^{14}C	^{3}H
Free compound:		
first crystallization	100	1320
second crystallization	120	1250
third crystallization	140	1280
Acetate:		
first crystallization	120	1250
second crystallization	140	1230

On the basis of these data we can conclude that both cholesterol and pregnenolone may be considered to be precursors of dehydroepiandrosterone. That cholesterol is a

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Abbreviation: ATP, adenosine triphosphate; ACTH, adrenocorticotropic hormone.

* This tissue was kindly made available to us by Dr. Louis J. Soffer and J. Lester Gabrilove, Mount Sinai Hospital, New York City.

precursor has been demonstrated previously[1]. The possibility of a conversion of pregnenolone to dehydroepiandrosterone has been suggested by LIEBERMAN[2] but this reaction has not been demonstrated previously. A likely pathway for the conversion might involve 17α-hydroxypregnenolone.

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Fractionation of calf-lens protein

Since 1894 when MÖRNER[1] reported that lens contains three soluble proteins, α-, β- and γ-crystallins, attempts have been made to obtain a definitive fractionation of soluble lens protein. Different results have been obtained by different methods of fractionation. Thus conventional electrophoretic techniques[2,3] have indicated the presence of two to three components; continuous-flow electrophoresis[4], 5 components; immunological studies[5,6] from five to eight components. Furthermore FRANÇOIS[7] has reported the fractionation of embryonic lens into ten fractions by means of ultramicro electrophoresis in agar gel.

A method has now been developed for the chromatographic fractionation on DEAE-cellulose* of the soluble protein of calf lens. By this procedure, 10 components have been isolated.

DEAE-cellulose (capacity, 0.8 mequiv./g) was prepared by washing with 0.5 M NaOH and water. The fine particles were decanted and the cellulose was suspended in 0.001 M phosphate buffer, pH 7.0. All further operations were performed in a cold room at 4°. The DEAE-cellulose column, prepared by the procedure of PETERSON AND SOBER[8], contained 16 g of adsorbant.

In a Potter-Elvehjem homogenizer, 1 g of calf lens was homogenized in 10 ml water. The lens homogenate was then centrifuged for 2 min at 11,000 × g and the insoluble material was discarded. The soluble lens protein was dialyzed against distilled water for 18 h, after which an equivalent of 250 mg (dry wt.) was added to the column.

The components were fractionated by stepwise elution. All buffers were prepared by appropriate dilution of 0.500 M (NaH_2PO_4, K_2HPO_4), pH 6.80 ± 0.01. Fractions were collected in 10-ml aliquots at the average rate of 40 ml/h. Only slight modification of the elution pattern was found when the flow rate was 100 ml/h. Regeneration was accomplished by washing the column with the following solutions: 180 ml

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* Purchased from Brown Co., Berlin, New Hampshire.
Abbreviation, DEAE-, diethylaminoethyl-.

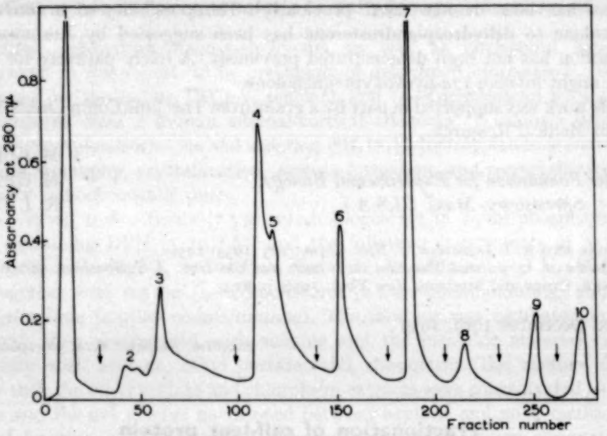

Fig. 1. Chromatographic pattern of 250 mg of soluble calf-lens protein applied to 16 g of DEAE cellulose. Stepwise elution was performed by successive application of the following buffers prepared from 0.500 *M* phosphate, pH 6.80: 0.001 *M*, 0.015 *M*, 0.030 *M*, 0.050 *M*, 0.080 *M*, 0.10 *M*, 0.40 *M*. The final eluting solution contained 0.20 *M* NaH_2PO_4 and 0.20 *M* NaCl. Arrows indicate the change in eluting buffer.

of 0.5 *M* NaOH, 160 ml of the 0.500 *M* phosphate buffer, 80 ml of 0.1 *M* phosphate buffer, and finally 500 ml of 0.001 *M* phosphate buffer.

Fig. 1 illustrates a typical fractionation of 250 mg (dry wt.) of calf lens. The fractionation was followed by the absorbancy at 280 mμ and by reaction with ninhydrin. Recoveries of 90% ± 5% have been obtained. The six major components represented by peaks 1,3,4,5,6 and 7 comprise 85.6% of the total protein recovered.

With this method lens protein can be fractionated at neutral pH in 65 h. Characterization of the protein components is in progress.

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The conversion of ubiquinone to ubichromenol

MORTON[1] has recently elucidated the structure of a substance, first described in 1953[2] as a constituent of the unsaponifiable matter of the livers of rats deficient in vitamin A, and has given it the name ubichromenol, because it is a hydroxychromene isomeric with ubiquinone. Ubichromenol has been reported to accompany ubiquinone in the kidneys and liver of many species[3].

The purpose of this note is to draw attention to the fact that adsorption of ubiquinone on alumina, a procedure used in the isolation from natural products of ubichromenol together with ubiquinone, brings about the partial conversion of ubiquinone to a product which appears to be identical with ubichromenol.

When a solution of pure ubiquinone(50)[*] in 0.5 ml petroleum ether (b.p. 60°–80°) was poured on to a column of alumina (Brockmann quality, purchased from Brocades, Stheeman & Pharmacia), 0.9 cm dia, the colour changed within 1 min from yellow orange to brown. After 24 h the pigment (now yellow brown) was extracted with acetone–10 % aq. HCl (9:1, v/v) and the absorption spectrum measured in ethanol. This showed maxima at 225 mμ, 275 mμ (with a shoulder at 282 mμ) and 329 mμ (*cf.* maxima at 233 mμ, 275 mμ, 283 mμ and 332 mμ in cyclohexane reported by MORTON[1]). The spectrum was not changed by addition of KBH_4. The compound was, however, oxidizable with $HAuCl_4$, which caused an increase in the absorption at 275 mμ.

The rate of conversion of ubiquinone into ubichromenol was decreased by pretreatment of the alumina with HCl, or by the addition of water (*e.g.* 4 %) and increased by lowering the concentration of ubiquinone adsorbed on the alumina (by partial elution with petroleum ether containing 4 % ether).

These findings must raise doubts as to the existence of ubichromenol in tissues. Solanachromene[7], also a chromenol, may also be a cyclic isomer of a natural quinone (KOFLER's quinone[8]) formed from the latter during isolation. (If this is the case the structure of solanachromene should be revised so that it has 8 instead of 9 isoprenoid units in the long side chain.)

A possible mechanism of the conversion of these quinones to hydroxychromenes is given in reactions (a) and (b) of Fig. 1. Attention should be given to the possibility that DIMTER's "hepene"[9] (which may be identical with MORTON's unsaturated hydrocarbon[10]) was also derived by the degradation of ubiquinone during isolation (reaction (c)).

Ubichromenol, which is formed by isomerization of ubiquinone, should not be confused with the acid-reduction product of ubiquinone (λ_{max}, 292 mμ (ethanol)), which we have suggested[4] is a chroman. A suitable name for this compound, which probably has the structure 3,4-dihydroubichromenol[11], is ubichromanol.

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[*] The preparation of ubiquinone used, like that previously described[4], was isolated from horse heart by a method essentially the same as that used by MORTON's group in their early work[5], except that pyrogallol was added during the extraction. A redetermination of the extinction coefficients of our first sample gave the following values: $E^{1\%}_{1\,cm}$ (ethanol) at 275 mμ, 165; after addition of KBH_4, $E^{1\%}_{1\,cm}$ (ethanol) at 290 mμ, 46.5. The m.p. of 48° previously reported[4] has been confirmed. The infrared spectrum, previously determined by Dr. P. J. VAN DER HAAK, showed none of the typical bands of the monoethoxy homologue at 10.10 μ and 11.18 μ, or of the diethoxy homologue at 8.51 μ, 10.20 μ and 11.05 μ. These measurements do not support the suggestion[6] that MORTON's isolation procedure yields ethoxy artifacts as the main product. The 8.30 μ, 8.67 μ and 10.55 μ bands ascribed to coenzyme Q_{10} by FOLKERS[6] were present in the spectrum of our product.

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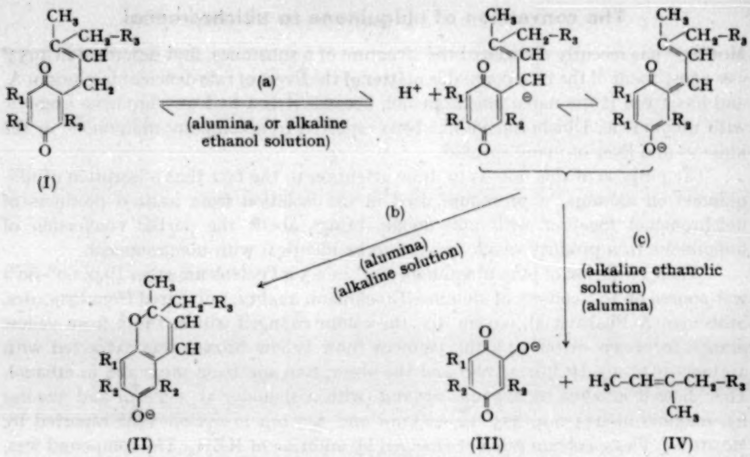
(I)
(a)
(alumina, or alkaline ethanol solution)
H+
(b)
(alumina)
(alkaline solution)
(c)
(alkaline ethanolic solution)
(alumina)
(II)
(III)
(IV)
+

Fig. 1. Possible mechanism of conversion of substituted allylbenzoquinones into 6-hydroxychromenes and "hepene" homologues.

I	II	IV	R_1	R_2	R_3
Ubiquinone(50)	Ubichromenol	Hepene	$-OCH_3$	$-CH_3$	$-(CH_2-CH=C(CH_3)-CH_2)_9-H$
Kofler's quinone	Solanachromene	$C_{45}H_{74}$	$-CH_3$	$-H$	$-(CH_2-CH=C(CH_3)-CH_2)_8-H$

Experiments are in progress to test whether other substituted allylbenzoquinones, *e.g.* trimethylphytylbenzoquinone, vitamins K_1 and K_2 are also converted to 6-hydroxychromenes by adsorption on alumina.

The author's thanks are due to Prof. E. C. SLATER for stimulating discussions and to Miss OLGA TOL for valuable assistance.

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Photoreduction of 2,4-dinitrophenol by chloroplasts

Recently we have shown that DNP is able to catalyse the generation of ATP by illuminated spinach chloroplasts[1]. We explained this effect provisionally by suggesting that DNP, just as the other compounds effective as cofactors in photosynthetic phosphorylation, acts as an intermediate electron carrier across some gap in the electron-transport chain of isolated chloroplasts. This would imply that DNP can be transformed by chloroplasts into a reversible oxidation-reduction system. We have now found that illuminated chloroplasts are able to reduce DNP to 2-amino-4-nitrophenol, and that the latter compound can serve as a cofactor of photosynthetic phosphorylation.

The reaction was carried out at 15° in an illuminated Warburg respirometer. Anaerobic conditions were maintained by flushing the Warburg manometer vessels with pure N_2 and introducing $CrCl_2$ in a side-arm. The reaction mixture consisted of 1 ml of a suspension of chloroplasts in 0.1 *M* Tris buffer, pH 7.5, containing 1 mg chlorophyll and prepared as described previously[2], 0.6 μmole DNP, and deionized water to give a final volume of 3.0 ml. The reaction was terminated after 60–90 min by turning off the light. Chloroplasts were removed by centrifugation, and the absorption spectrum of the orange supernatant was determined in the Unicam spectrophotometer. A rather broad absorption band was observed with a maximum at 430–440 mμ. The absence of DNP in the supernatant was indicated by the complete disappearance of the characteristic absorption maximum of this compound at 360 mμ.

The transformation of DNP proved to be dependent on light and on anaerobic conditions. When the mixture was kept in the dark or when the chloroplasts were illuminated in air, DNP could be recovered nearly quantitatively even after 2 h. This was also the case when boiled chloroplasts were illuminated in the presence of DNP. The transformation of DNP proceeded approximately twice as fast if phosphorylating reagents (phosphate, ADP, $MgCl_2$, glucose and hexokinase) were present. The reaction was strongly inhibited by 10^{-4} *M* *o*-phenanthroline, $4 \cdot 10^{-6}$ *M* 3-(4-chlorophenyl)-1,1-dimethylurea and 10^{-3} *M* hydroxylamine, which are known to inhibit the Hill reaction, and was accompanied by oxygen evolution (1.35 μmole of O_2 per μmole of DNP). This suggests that DNP serves as a Hill oxidant and can be reduced by illuminated chloroplasts. As $3 \cdot 10^{-4}$ *M* *p*-chloromercuribenzoate showed no inhibitory effect, we think that photoreduction of TPN, which is very sensitive to this poison, is not involved in this process.

In order to identify the product of the photoreaction, we collected the contents of a great number of Warburg vessels in which the reaction had been performed, and removed the chloroplasts by centrifugation. The supernatant was acidified to pH 5 by addition of 1 *N* H_2SO_4, and extracted with ether. The ethereal extract was thoroughly extracted with NaOH solution of pH 9. The aqueous solution was then again brought to pH 5 by addition of 1 *N* H_2SO_4, extracted with ether, and the ether was removed by vacuum distillation. The residue was recrystallized twice from water, and the crystalline solid (needles) dried in a vacuum desiccator over conc. H_2SO_4. The orange-yellow crystals melted at 143°. The melting point was not depressed by mixing the substance intimately with synthetically prepared 2-amino-4-nitro-

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Abbreviations: DNP, 2,4-dinitrophenol; FMN, flavin mononucleotide; ATP, adenosine triphosphate; ADP, adenosine diphosphate; TPN, triphosphopyridine nucleotide; Tris, tris-(hydroxymethyl)aminomethane.

phenol[3]. The compounds were found to be identical also with respect to ultraviolet and infrared spectra, and to paper chromatography in four different solvents.

2-Amino-4-nitrophenol was shown to be capable of catalyzing photosynthetic phosphorylation. We therefore may conclude that the ability of DNP to catalyze ATP synthesis by illuminated chloroplasts is due to photoreduction of this compound.

We have found that photoreduction of DNP did not occur in the presence of phosphorylating agents and vitamin K_3 or FMN. This indicates that the transformation of DNP into 2-amino-4-nitrophenol cannot account for the insensitivity of photosynthetic phosphorylation to DNP. It seems evident, therefore, that DNP itself is unable to effect uncoupling of photosynthetic phosphorylation.

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Announcement

Vth International Congress of Biochemistry

The Vth International Congress of Biochemistry, organized by the U.S.S.R. Biochemical Society under the auspices of the International Union of Biochemistry, is to be held from August 10th–16th, 1961, in Moscow. Details concerning this Congress may be obtained from the Secretary-General of the Organizing Committee, Professor N. M. Sissakian, Leninsky prospekt 33, Moscow B-71, U.S.S.R

KINETIC STUDIES OF TEMPERATURE EFFECTS ON THE CELLULAR LEVEL*

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SUMMARY

Temperature and illuminance dependence of growth was studied in the low- and high-temperature strains of the green unicellular alga, *Chlorella pyrenoidosa*. The developmental and metabolic diversity of cells in steady-state microbial populations was evaluated. The effects of temperature and light were considered separately on the accumulation of cell material and on cell division. It was concluded that the values of activation energy for growth are characteristic of neither of these two processes but are influenced by both of them. The implications of the master reaction theory and of the statistical approach on the cellular and reaction levels in biochemical and biophysical studies were critically discussed.

INTRODUCTION

Kinetic studies of temperature effects on simple reactions or complex biological processes, *in vitro* or *in vivo*, have been invariably evaluated on the reaction level. The enormous volume of the literature in this field precludes discussion here of even the most significant publications. A reference is made to several books and reviews in which the effects of temperature on life processes, the kinetics of these processes and the theoretical aspects of the reaction rates are discussed[1–5].

A treatment of the temperature data on a cellular level, attempted in this report, may add a new approach in kinetic studies of biological processes. By using unicellular organisms all complications arising from the structural and physiological relationships of the cells in multicellular organisms were avoided. Photoautotrophic properties of these organisms permitted studies of temperature effects in connection with another environmental factor, light. The availability of the strains of different temperature preference allowed an extention of the temperature range not readily obtainable with only one organism.

MATERIAL AND METHODS

The studies involved comparative investigations of two strains of green unicellular alga: *Chlorella pyrenoidosa*, Emerson strain, representative of the low-temperature

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[*] Scientific Article A751, Contribution No. 3011 of the University of Maryland Agricultural Experiment Station.

group, and *Chlorella* 7-11-05 representing the high-temperature algae. Descriptions of the strains and details of the technique have been published elsewhere[6,7].

RESULTS AND DISCUSSION

Values of activation energy for growth

The dependence of growth in two strains on temperature is described at two light intensity levels: 440 foot candles, fluorescent lamps, and 1600 foot candles, incandescent lamps (Fig. 1). In an attempt to evaluate the temperature curves the ascending portions of the curves in Fig. 1 are replotted as logarithms of the rates against the reciprocals of absolute temperature (Fig. 2). Although these curves were plotted in Fig. 2 with the abrupt breaks in the slopes, these sharp breaks are considered not to be real but to be caused by the relatively small number of measurements along the temperature scale, the actual changes in the slopes being gradual.

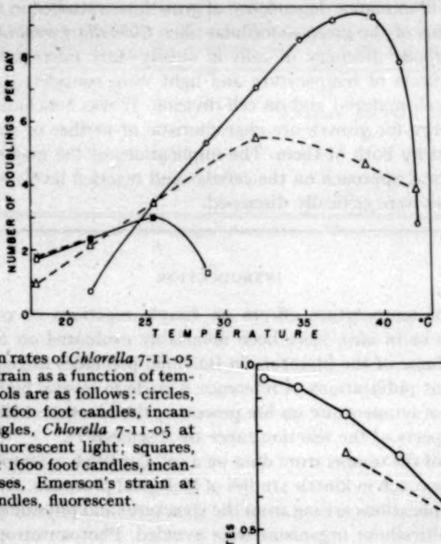

Fig. 1. Multiplication rates of *Chlorella* 7-11-05 and the Emerson strain as a function of temperature. The symbols are as follows: circles, *Chlorella* 7-11-05 at 1600 foot candles, incandescent light; triangles, *Chlorella* 7-11-05 at 440 foot candles, fluorescent light; squares, Emerson's strain at 1600 foot candles, incandescent; and, crosses, Emerson's strain at 440 foot candles, fluorescent.

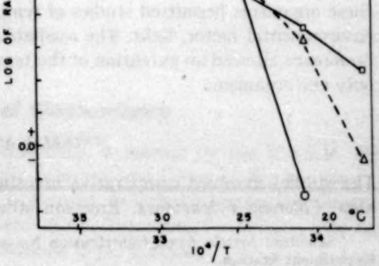

Fig. 2. Logarithms of the multiplication rates for *Chlorella* 7-11-05 and the Emerson strain plotted against the reciprocals of absolute temperature. Circles, *Chlorella* 7-11-05 at 1600 foot candles; triangles, *Chlorella* 7-11-05 at 440 foot candles; and squares, Emerson's strain at 1600 and 440 foot candles.

A comparison of the logarithmic curves indicates that their slopes are steeper and consequently the values of activation energy, μ, calculated from these slopes (Table I), are higher for *Chlorella* 7-11-05 than for the Emerson strain, compared in the same temperature ranges. For *Chlorella* 7-11-05 they are greater at 1600 foot candles than at 440 foot candles. Since growth curves are downwardly concave, the values of μ increase with the decrease in temperature. For *Chlorella* 7-11-05 in the temperature range of 21.5–25.5° at 1600 foot candles μ is equal to 68,000.

TABLE I

VALUES OF ACTIVATION ENERGY (μ) FOR GROWTH

Temperature interval, °C	*Emerson at 440 and 1600 f.c.*	*7-11-05 at 440 f.c.*	*7-11-05 at 1600 f.c.*
18.0–21.5	15,000	41,000	
21.5–25.5	12,000	21,000	68,000
25.5–28.9		13,000	23,000
28.9–32.1			17,000
32.1–34.9			12,000
34.9–37.0			6,000

Far-reaching conclusions could be drawn from the kind of data presented in Fig. 2 and in Table I. The conclusions and speculations could bear upon the pronounced difference in the levels of activation energy for growth between the low- and high-temperature strains and upon the higher requirements for activation energy at higher light intensity clearly demonstrated for *Chlorella* 7-11-05. From the inconsistency of the slopes of the logarithmic curves in different temperature intervals it could be deduced, as it has been often done, that different reactions limit the rate of the over-all process at different temperatures. An attempt to determine the number of these reactions and to formulate their thermodynamic and possibly some other characteristics could also be made.

The inconsistency and differences in the steepness of the slopes of the logarithmic curves require, however, special consideration. Given a sufficient temperature range any curve bends at the upper end. The cause of the phenomenon is probably a competing process, or possibly a number of competing processes, which interferes with the increase in metabolic rates with the increase in temperature. Such processes can operate at temperatures considerably lower than those for optimal growth. But this is hardly adequate to explain the gradual decrease in the logarithmic growth rates all the way to temperatures at which the growth rate is minimal. An examination of the effects of temperature and light on cell division may provide such an explanation.

The effect of temperature and light on cell division

The effect of temperature on cell division is indicated by the following observation. If a suspension of synchronized cells of *Chlorella* 7-11-05, grown in the light and brought to readiness for cell division, is placed in the dark at 39°, cell divisions in individual cells are completed within 3 to 4 h. If the same algal suspension is placed in the dark at 15°, cell division proceeds so slowly that even after 24 h in darkness one finds only a few cells divided into daughter cells. Between 15° and 39° the rate of cell division is a function of temperature. At lower temperatures the over-all growth of an algal suspension is greatly retarded by the effect of low temperature on cell division. This explains why the logarithmic curve of the growth rates (Fig. 2) is steeper and the value of μ higher in the lower temperature range.

Another factor contributing to the change in the slopes of the curves plotting logarithms of the growth rates against temperature (Fig. 2) is the effect of light intensity on cell division. In a suspension of synchronized cells of *Chlorella* 7-11-05 cell divisions usually start after 9 to 10 h of growth under optimal conditions of temperature and light intensity. However, when the time for cell division arrives all cells do not divide simultaneously. Individual cells divide after different periods of growth, and, in an algal suspension as a whole, cell divisions are generally spread over 8 to 9 h if the cell suspension is left in the light. If a suspension of cells ready to enter cell division is transferred into darkness, then the time necessary for all cells to complete division is shortened to 3–4 h.

The effect of light intensity on cell division is reflected in higher requirements for minimal temperatures at higher light intensities if cell division and over-all growth are to take place. In Fig. 3 the lower portions of the growth curves of Fig. 1 are extrapolated to limiting temperatures at which multiplication rates have their minimal values. For *Chlorella* 7-11-05 the limiting temperature for growth is at 1600 foot candles at 20.5° and at 440 foot candles it is at 15.3°. For the Emerson strain the minimal temperatures for growth at these light intensities are, respectively, 7.8° and 6.6°. As a result the starting points for growth are higher on the temperature scale at higher light intensities and the temperature curves become steeper. This causes values of μ to rise at higher light intensities (Table I).

Master reaction theory

An attempt to interpret the effects of temperature and light intensity on growth can be advantageously connected with the understanding of growth as consisting of two processes—cell division and accumulation of cell material. A conception of the dependence of the processes of accumulation of cell material and cell division on temperature and light intensity is given in Fig. 4 in which logarithms of the hypothetical rates of these two processes at two levels of light intensity are plotted against the reciprocals of absolute temperature.

In photoautotrophic organisms the process of accumulation of cell material is largely dependent on photosynthesis. Both the increase in temperature and in light intensity, within limits, favorably affect the photosynthetic rate, and, at higher temperatures, the light saturation point is moved to a higher light intensity. For this reason the dependence of the rate of the accumulation of cell material on temperature is depicted (Fig. 4) at two light intensities by two diverging lines indicating a favorable effect of higher light intensity at higher temperatures.

It has been shown that the increase in temperature, within limits, favorably affects the rate of cell division, but the increase in light intensity, at least at low temperatures, has an unfavorable effect on this process. For this reason the lines indicating the rate of cell division at two light intensities are drawn far apart at lower temperatures. The suggestion that these two lines are converging, as they approach higher temperatures, needs further experimental verification.

The difference in the slopes of the temperature curves for the accumulation of cell material and cell division results in a crossing of the curves at some temperature. With regard to the effects of temperature one is tempted to discern two master reactions governing the over-all process of growth in the lower and higher temperature ranges. Each of these reactions is connected with one of the two different processes—one, cell division, limiting the over-all process of growth in the lower temperature range; another, accumulation of cell material, the rate of which is largely responsible for the rate of the over-all process of growth in the higher temperature range.

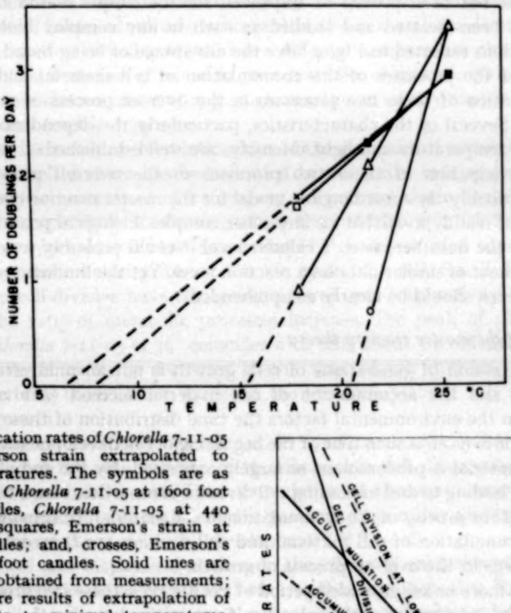

Fig. 3. Multiplication rates of *Chlorella* 7-11-05 and the Emerson strain extrapolated to minimal temperatures. The symbols are as follows: circles, *Chlorella* 7-11-05 at 1600 foot candles; triangles, *Chlorella* 7-11-05 at 440 foot candles; squares, Emerson's strain at 1600 foot candles; and, crosses, Emerson's strain at 440 foot candles. Solid lines are based on data obtained from measurements; broken lines are results of extrapolation of experiment data to minimal temperatures.

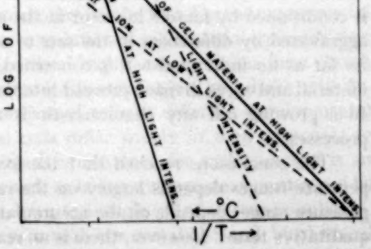
LOG OF RATES
CELL DIVISION AT LOW LIGHT INTENSITY
ACCUMULATION OF CELL MATERIAL AT HIGH LIGHT INTENS.
ACCUMULATION AT LOW LIGHT INTENS.
DIVISION AT HIGH LIGHT INTENS.
←°C
1/T→

Fig. 4. Hypothetical dependence of the accumulation of cell material and cell division on temperature and light intensity.

From Fig. 2 one can see that the most drastic changes in the slopes of the logarithmic curves for growth coincide with the temperature interval of 25–26°. These changes are reflected in differences of μ values below and above this point. For *Chlorella* 7-11-05 at 1600 foot candles μ for the temperature range of 21.5–25.5° is

68,000, and for that of 25.5–28.9°, 23,000; at 440 foot candles the values of μ are, respectively, 21,000 and 13,000. In accordance with the view that a master reaction can be characterized by the value of activation energy, one logically concludes that for *Chlorella* 7-11-05 the temperature region of 25–26° is a breaking point at which a change from one master reaction to another takes place.

The master reaction theory worked out by BLACKMAN[8] and PÜTTER[9] and expanded by CROZIER[10] is a speculative theory as applied to complex biological processes. It has not been proved or disproved for the simple reason that no master reaction has been isolated and studied as such in any complex biological process. The speculations reflected in Fig. 4 have the advantage of being based on the factual knowledge of the processes of the accumulation of cell material and cell division. The participation of these two processes in the over-all process of growth is not a speculation. Several of the characteristics, particularly the dependence of these two processes on temperature and light intensity, are well established.

The participation of these two processes in the over-all process of growth represents probably the most tangible model for the master reaction concept and such a model is not readily available for any other complex biological process. Though the discussion of the data here is on a cellular level it could probably serve as a pattern for the treatment of similar data on a reaction level. Yet the limitations of the master reaction concept should be clearly comprehended.

Evaluation of the master reaction theory

In a succession of generations of cells growth is not an uninterrupted process. Cell division and the accumulation of cell material succeed each other in time. Depending on the environmental factors the time distribution of these two processes in the life cycle of a cell is such that at the beginning of cell development the accumulation of cell material is predominant or largely expressed. By the end of the life cycle the processes leading to and indicating cell division become dominant, to the complete suppression of the process of the accumulation of cell material. In substituting for each other the accumulation of cell material and cell division are consecutive and interdependent steps in the over-all process of growth.

During a more or less extended period of the life cycle these two processes proceed simultaneously and compete with each other. Competition between these two processes is conditioned by factors inherent in the genetical constitution of the organism. It is aggravated by differences in the sets of conditions favoring each of these processes. As far as an individual cell is concerned, the processes of the accumulation of cell material and of cell division proceed interdependently, consecutively, and competingly. In a growing cell any characteristic is a resultant of the interplay of these two processes.

The conclusions reached that the over-all process of growth in the lower temperature ranges depends largely on the rate of cell division, and in the higher temperature ranges depends on the accumulation of cell material may be useful in broad qualitative terms. However, there is no reason to believe that at any temperature one of these two processes assumes an exclusive role as a limiting factor in the over-all growth process. The slopes of the temperature curves and the values of μ are characteristic of neither of these processes but are influenced by both of them. The breaks in the slopes of temperature curves are uncertain indications of the temperatures at which one of these processes assumes a predominant role in conditioning the rate of growth.

For photoautotrophic organisms temperature dependence is complicated by the complementary effects of light. For organisms growing in the dark, such as *Escherichia coli*, temperature curves were obtained giving a linear Arrhenius plot over an extended temperature range[9]. However, this observation does not indicate that in heterotrophic organisms the processes of cell division and of the accumulation of cell material are not affected differentially by temperature. Experiments on the synchronization of bacterial cultures indicate that also in these organisms cell division and the accumulation of cell material have different temperature characteristics[11]. Thermodynamic information obtained in growth studies of heterotrophic organisms is therefore no more characteristic of any single reaction or of any simple process than it is for photoautotrophic organisms.

So far the discussion of the relationships of the processes of accumulation of cell material and of cell division has been limited to the status in a single cell or in a succession of a few generations of its progeny. In multicellular organisms as well as in steady-state suspensions of microorganisms the situation is much more complex.

Studies on synchronized algal suspensions[12–14] have revealed considerable differences in metabolic activity during the life cycle of a cell. Small daughter cells emerging from cell division have the lowest rates of metabolic activity. Then, as the cells grow, the rates of metabolic processes increase. The peak of photosynthetic activity in *Chlorella* 7-11-05 at 39° coincides with cells about 3 h old after which time the photosynthetic capacity decreases to the time of cell division. The peak in respiration activity occurs much earlier in the life cycle—about half an hour after the start of growth.

Studies on the formation of nucleic acids[15,16] indicated considerable differences in the time course of the accumulation of the ribonucleic and desoxyribonucleic acids during the life cycle of the cells. The accumulation of chemical elements as well as effect of the deficiency in different elements, particularly in sulfur, also has been shown to be dependent on the stage of cell development[17,18].

Biochemical and biophysical studies on the effects of environmental factors on cellular characteristics have been based on a tacit assumption that these effects on the studied cellular characteristics are the same in sign and more or less the same in degree in different cells. No proof for this assumption can be found in the experimental data. As was pointed out, cell division and accumulation of cell material have different optimal temperature, illuminance, and nutritional requirements. It has been shown that cells at different stages of the life cycle have different rates of metabolic activity. Since a steady-state cellular population consists of cells in different developmental stages, it can be expected that individual cells differ widely in their responses to environmental factors. Any cellular characteristic obtained for such material is an average of characteristics of individual cells and, as in any average, may or may not be intrinsic to any portion or, strictly speaking, even to a single cell of a given population. Studies on synchronized microbial cultures reveal the metabolic and biochemical differences in cells of different developmental stages and emphasize the futility of the statistical approach in measurements of cellular characteristics for steady-state populations.

All this poses a problem in the calculation of theoretical curves from kinetic data for complex biological processes. Calculations of the theoretical curves involve reliance upon one of the theories of reaction rates. However, it should be pointed out that whatever major theory—the Eyring theory[19] of absolute reaction rates, or the Michaelis-Menten theory[20] of the kinetics of enzymic reactions—is laid as a basis for calculations of the reaction rates, the member of the equation indicating temperature dependence of the process is obtained in practice from kinetic data derived from measurements of the rate of the process at different temperatures. Any agreement of the theoretical curve with experimental data is often considered a proof of the validity of the theoretical curve. Thus, from both the theoretical and the experimental point of view a proper evaluation of kinetic data is of considerable interest.

ACKNOWLEDGEMENTS

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A REACTION OF WATER WITH RAT-TAIL TENDONS (HYDRATION-ELONGATION)

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(Received May 5th, 1959)

SUMMARY

1. The rate of lengthening of a dry tendon was analyzed when water, water–salt, and water–alcohol solutions were added.

2. An empirical-mathematical treatment of the experimental data resulted in a certain constant (K_{HE}) which could be related to the temperature ($1/T$), dielectric constant (D) and ionic strength (μ) of the water solutions.

3. The experimental findings were compared with recent knowledge of the interaction of water with proteins. Also, the influence of the solvent was examined in light of the possible existence of electrostatic stabilization forces in the collagen-mucopolysaccharide interaction of tendons.

INTRODUCTION

It is recognized widely that water has an important role in biological systems. In a recent review of this subject KLOTZ[1] supported the idea that "lattice" water was bound intimately to proteins. This was suggested to explain the masking of functional groups of protein *molecules in solution.*

Collagen is a fibrous protein in close union with water, carbohydrates and amino sugars (mucopolysaccharides). According to FESSLER[2] mechanical stress, shock, and trauma might be transmitted through sets of "sponges" composed of water-mucopolysaccharide molecules and the fibrous constituents. These sponges also could account for the viscoelastic properties of connective tissue and other hydrated systems. The central theme of the studies in our laboratory is to determine how the elasticity and swelling of rat tail tendons might be influenced by water. From this it may be possible to study the cross-linking reactions which collagen appears to undergo as a result of the process of aging[3-5]. It is the purpose of this report to present a mechanico-chemical analysis of the *hydration-elongation* of tendons and to study the effect of mixed solvents and salt solutions upon this process.

EXPERIMENTAL

Preparation of tendon

Tendons were removed from rat tails after the skin was cut the full length with scissors and peeled away by hand. A break between the vertebrae was made with a pair of pliers and the attached tendons were pulled from the rest of the tail. Proceeding from the tip, tendons were removed successively. Lengths shorter than 12 cm were not used. The collection of tendons was washed 3 times with 50-ml portions of 0.1 *M* sodium chloride, then washed with distilled water for 30 min. They were removed, disentangled, and dried. Tendons dried to a constant weight/length (W) within 30 min following exposure to the atmosphere of the laboratory (22° to 23° temperature and 75 % relative humidity). More extensive drying was attempted but subsequent handling caused some rehydration thereby re-establishing the original dry weight/length ratio (W).

Apparatus

The tendon was inserted into a 20-ml syringe cylinder through the bore of the glass tip; one end was immobilized by forcing an adapter (2 or 3-way) over the tip while the other end, 10 cm distant, was attached to a mechanical lever system by a small "alligator" clip. A meter stick was suspended and balanced on metal knife edges and provided a writing lever arm (70 cm long) and a driving arm (3.5 cm long) which caused a 20× magnification. Records of length *vs.* time were obtained by an electrically driven kymograph. The system was unbalanced slightly so that there was a positive load of 20 g on the tendon. A vacuum line was attached to one of the ports of the adapter on the syringe and air was sucked through the cylinder. This facilitated removal of liquids and was a satisfactory means of drying the tendon between repeated runs.

Hydration

The tendon was hydrated by rapidly (1–2 sec) admitting liquid of volume sufficient to cover it. The liquid that was added and the syringe-tendon assembly were held at constant temperature in a thermostated water bath. The temperature dependence of the hydration-elongation was studied in water bath temperatures ranging from 10 to 60°.

Dehydration

The temperature of dehydration was varied by using a hair dryer to force air into the cylinder. Regulation was achieved by alternating the heater switch from ON to OFF while the exact temperature was indicated by a thermometer placed alongside the tendon in the cylinder. In this manner the drying and concomitant shortening of the tendon was at air temperatures ranging from 20 to 100°. The influence of the rate of delivery of air at a fixed temperature was measured. The current to the fan of the dryer was varied by using a Variac and the shortening was measured at Variac settings from 110 to 0 at a temperature of 23°.

Influence of tendon thickness

The influence of tendon thickness was studied by measuring the hydration-elongation of specimens having W values from 0.1 to about 0.6 mg/cm.

Mixed liquids

The hydration-elongation was studied in water and its binary mixtures with methanol, ethanol, isopropanol, and propanol. The composition of the mixes ranged from 0 to 100% alcohol. All of these studies were done at a temperature of 23°. Dielectric constant data were obtained from the literature[6] for the solutions described.

Salts

The influence of ionic strength of sodium chloride was studied by making up solutions ranging from 0 to 1.0 *M*. Four tendons with W values ranging between 0.2 and 1.0 mg/cm were used for this study.

RESULTS

Fig. 1 shows a typical length–time curve. Upon the addition of water the length ($L_0 + L$) of the tendon increased from the initial "dry" value (L_0) to a steady-state value ($L_0 + L_\infty$) in about 1 min. At this point the water was removed and air was sucked through the cylinder. The tendon was dried at a temperature of 23° until it ceased to shorten. The "dry" length was called L_0 and the maximum increase in length (L_∞) was a distance of about 2–4 mm. The length *vs.* time curves produced by subsequent addition of water followed by drying could be reproduced at least 10 times.

There was a progressive increase in shortening when the dehydration was conducted at higher air temperatures. The extent of shortening was constant for Variac settings from 110 to 80. Below this the rate of delivery of air became rate limiting and inadequate drying resulted.

Fig. 2 shows the shape of length *vs.* time curves obtained when the drying temperature was increased from 21 to 100°. $L_0 + L_\infty$ did not change but L_0 was reduced. A particular length *vs.* time curve could be reproduced when the temperature of the hydrating liquid and the air-dehydration were reproduced. The two temperatures need not be the same. Once a tendon was heated a curve previously obtained at a lower temperature was shifted to high length when the elongation was done at the lower temperature. In this way the dehydration was not reproducible. But, if the temperature of the two steps were repeated the hydration-elongation and dehydration-shortening could be reproduced.

The rate of elongation ($\Delta L/\Delta t$) was calculated and appears as a function of *t*

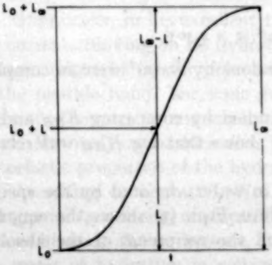

Fig. 1. A typical length–time curve illustrating the hydration-elongation of rat tail tendons. The symbols identify those aspects of the process which are used in the analysis presented in the text.

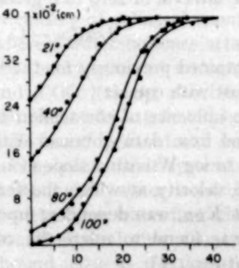

Fig. 2. The influence of dehydration-temperature upon the subsequent hydration-elongation conducted at 25°.

in Fig. 3. The maximum in the velocity of elongation always was sharp. When the tracings were made at very fast speed the break in the curve continued to be sharp.

The velocity ($\Delta L/\Delta t$) was plotted against ($L_\infty - L$) and curves were obtained that were similar to those shown in Fig. 3. However, Fig. 4 shows that plots of $\Delta L/\Delta t$ *versus* the product $(L_\infty - L)t$ were linear with a positive slope (K_{HE}). This empirical observation formed the basis for eqn. (1).

$$\mathrm{d}L/\mathrm{d}t = K_{HE}(L_\infty - L)t \tag{1}$$

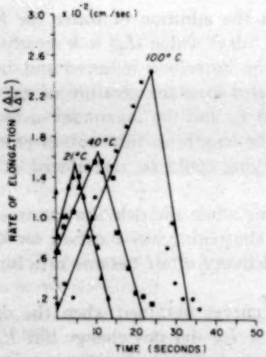

Fig. 3. An empirical analysis of the velocity of elongation as a function of time and dehydration temperature.

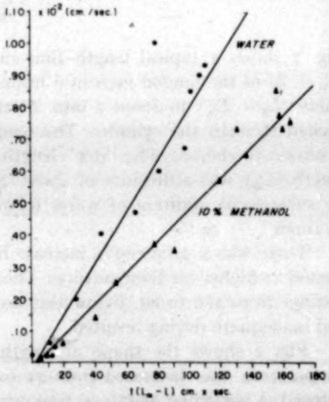

Fig. 4. The linear relationship between velocity of elongation and the quantity $t(L_\infty - L)$ as influenced by water and 10% methanol.

Here, $\mathrm{d}L/\mathrm{d}t$ is the rate of change of the length of tendon during the hydration, K_{HE} is a specific rate constant for the process, L_∞ is the maximum increase in length attained, and L is the change in length at any particular time, t. Rearrangement of the terms in equation (1), followed by integration between the limits of 0 to L in the time interval of zero to t, gives eqn. (2).

$$\mathrm{Log}(L_\infty - L) = \log(L_\infty) - K_{HE}t^2/(2 \times 2.303) \tag{2}$$

Data obtained previously for the elongation of tendons by water[7] were in complete agreement with eqn. (2).

The influence of the tendon thickness was studied by comparing K_{HE} and W evaluated from data obtained with water. Fig. 5 shows that log K_{HE} was related linearly to log W with a slope of -1.

The velocity at which the tendon elongated in water, denoted by the specific constant K_{HE}, was dependent upon the temperature. Eqn. (3) shows the equation which was found to relate the constant K_{HE} and the reciprocal of the absolute temperature ($1/T$).

$$\mathrm{Log}\ K_{HE} = A - B\ (1/T) \tag{3}$$

The hydration-elongation of tendons induced by binary liquid systems showed some interesting effects. Plots of log ($L_\infty - L$) *versus* t^2 for alcohol–water solutions showed an initial downward curvature which eventually became linear with t^2. When K_{HE} was evaluated from this linear region it could be related to the dielectric constant of the medium by eqn. (4). The methanol–water system deviated the least from eqn. (2) and was in agreement with eqn. (4) from 0 to 40 % methanol.

$$\text{Log } K_{HE} = \log (K_{HE})_0 + C\,(D - 1)/(2D + 1) \qquad (4)$$

In the non-linear region of eqn. (2) there appeared to be a systematic way in which the deviations occurred. Values of t^2 which reduced the y-ordinate to one-half (½) the value at zero time were estimated from graphs of log $(L_\infty - L)$ *versus* t^2. The values of $t^2_{1/2}$ for solutions with a dielectric constant of 50 formed the series Me (2000), Et (380), Isopro (200), and Pro (100).

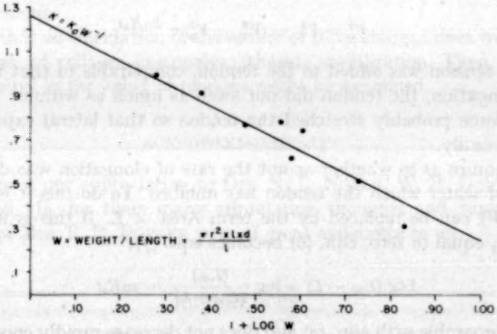

Fig. 5. The dependence of the specific constant (K_{HE}) upon the size (W) of rat-tail tendons.

Eqn. (5) shows the relationship that was used to relate the rate constant (K_{HE}) to the ionic strength (μ).

$$\text{Log } K_{HE} = \log (K_{HE})_{\mu = 0} - E(\sqrt{\mu}) \qquad (5)$$

DISCUSSION

Water

Gustavson, in his excellent treatise on the chemistry of collagen[8], summarized the current thinking on its hydration. The sites wherein water becomes attached to collagen might be (a) the polar side chains and (b) the oxygen and nitrogen atoms of the peptide bond. The ionic groups (NH_3^+) and (COO^-) might also bind water but since they are so few in number the hydration cannot be attributed largely to them. Mucopolysaccharides also bind water and Fessler[1] pointed out important viscoelastic properties of the hydrated collagen-mucopolysaccharide system.

According to Kanagy[9], Wollenberg[10], and Rougvie[11] water is adsorbed by collagen in a succession of steps. The most reactive sites attach water first while those of less binding ability become involved later. Bull[12] showed that about 21 % of the water of hydration in collagen was tightly bound whereas the 79 % of water subsequently added forced the chains apart so that their separation increased from 10 Å to 15–16 Å. The discontinuous adsorption of water probably holds for mucopolysaccharides as well.

In the dehydration step of the system which we described here the tightly held water probably was not removed. The subsequent addition of water, which permitted the hydration-elongation to occur, conceivably allowed the collagen-mucopolysaccharide chains of tendons to separate. At high drying temperatures more water was removed and tendons shortened more than at low drying temperatures.

Rat tail tendons undergo considerable swelling when placed in water. A model-mechanism that was consistent with the volume–time data was proposed by ELDEN[13] to account for the swelling of hide powder collagen and rat tail tendons. Eqn. (6) shows the proposed relationship between the initial volume (V_0), the final volume (V_∞), a specific swelling constant (K_s), a constant characteristic of the ability to resist any increase in volume (a), and the accumulated time of swelling (t).

$$V^2 = V_\infty^2 - (V_\infty^2 - V_0^2)\, e^{-2aK_st} \tag{6}$$

When a 20-g tension was added to the tendon, comparable to that involved in the hydration-elongation, the tendon did not swell as much as without the load[14]. The longitudinal force probably stretched the tendon so that lateral expansion was not accomplished easily.

Let us inquire as to whether or not the rate of elongation was dependent upon the amount of water which the tendon has imbibed. To do this it is assumed that the volume (V) can be replaced by the term Area $\times$ L. If this is inserted for V_∞ and V with V_0 equal to zero, eqn. (6) becomes eqn. (7).

$$\text{Log}\,(L_\infty - L) = \log \frac{(L_\infty)}{(L_\infty + L)} - 2aK_st \tag{7}$$

This is not comparable with eqn. (2) and does not decrease rapidly enough to coincide with the experimental points. Thus, it appears that there is a rapid reaction between water molecules and those centers which hold the tendon in its shortened state. This reaction goes to completion rapidly and the subsequent imbibition of water by swelling has no effect on the elongation.

Mixed solvents and salt solutions

The influence of solvent properties on the rate of simple ion-ion, ion-dipole, and dipole-dipole reactions in dilute solutions can be expressed by eqn. (4). When the dielectric constant becomes small, positive deviations of experimental data occur and this has been attributed to participation of the solvent in the reaction[15].

Specific solvent effects do appear to be involved when alcohol–water solutions were used for hydration-elongation. The deviation of the plots according to eqn. (2) became progressively greater in comparison to the series Me < Et < Isopro < Pro alcohols. Likewise, the electronegativity around the hydroxyl group[16], heats of hydration[17], and VAN DER WAALS' constants also increase in the same order. We do not know what part of the collagen-mucopolysaccharide system has an affinity for these alcohols.

The equations which show the dependence of K_{HE} on (D), and (μ) are identical in form to those kinetic expressions which account for the rate of interaction of charged molecules in solution[15]. These findings suggest that analogous electrostatic processes might participate in the solvent-induced lengthening of tendons.

These findings agree with those of BENSUSAN who measured the rate of formation of collagen fibers from soluble molecules[18]. The rate depended upon the concentration of alcohols used to change the dielectric constant. In the concentration region of inhibition the alcohols effectiveness increased in the order Me $<$ Et $<$ Isopro $<$ *tert.*-Butyl $<$ Pro. Increased ionic strength of simple electrolytes also decreased the rate of fiber formation.

At the isoelectric point of native collagen (pH 7) there are equal numbers of cationic and anionic charges. However, there could be an un-equal distribution of charges in a local region which could attract, or repel, similar excess local charges. The close proximity of mucopolysaccharides to collagen favors this situation because sulfonic acid groups of the former are dissociated at neutral pH. This could regulate the rate and extent of hydration-elongation by restricting, or enhancing, the separation of these chains.

It is hoped that an elucidation of the nature of these charged sites will contribute to our knowledge of collagen-mucopolysaccharide stabilization. Then, the participation of these sites in the aging of tendons might be examined.

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A MICROMETHOD FOR THE COLORIMETRIC DETERMINATION OF N-ACETYL GROUPS IN ACID MUCOPOLYSACCHARIDES

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SUMMARY

Deacetylation of mucopolysaccharides and other N-acetylated compounds with 2 *N* HCl–methanol is a useful and simple procedure for determining N-acetyl groups in these compounds. The acetyl groups are converted to methyl acetate which is colorimetrically determined as a hydroxamic–ferric complex. This method permits the determination of N-acetyl groups in a range of 1 to 10 μmoles and is more sensitive than previously described methods. The reproducibility and some of the factors affecting the deacetylation and colorimetric analysis have been studied.

INTRODUCTION

The determination of acetyl groups is of considerable importance in the study of mucopolysaccharides. Available methods are tedious and require relatively large amounts of material. Earlier procedures have been comprehensively reviewed by MEYER[1]. More recently a number of modifications including variation of conditions of hydrolysis and methods of determination of liberated acetic acid[2,3] have been studied.

This paper describes the quantitative microdetermination of acetic acid present as an N-acetyl group in the hexosamine moiety of certain mucopolysaccharides, amino acids and aromatic amines. The methyl acetate formed by acid methanolysis is distilled *in vacuo* and determined colorimetrically by transformation to acetohydroxamic acid which yields a purple color with ferric salts. The intense color of the hydroxamic–ferric complex permits determination of acetyl groups in the range of 1 to 10 μmoles. The residue after deacetylation can be used for further analyses or isolations. This method is relatively simple and rapid.

EXPERIMENTAL

Apparatus

Fig. 1 illustrates the distilling apparatus. It consists of a chamber (A) with a side arm (B) so bent that the latter extends to about 20 mm above the bottom of a suction tube (C). This suction is connected to a desiccator tube filled with $CaCl_2$. Between the desiccator tube and the water pump there is a T tube which can be opened to the outside.

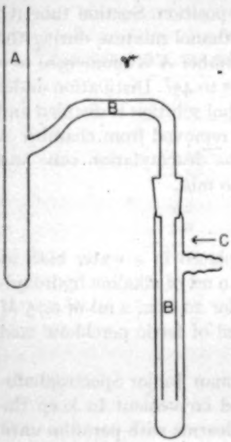

Fig. 1. Apparatus for the colorimetric microdetermination of N-acetyl groups. Tube A is 190 × 11 mm. All other dimensions are drawn to scale. A Polyethylene cap is used for chamber A, but a rubber stopper is preferable for chamber C.

Reagents

(a) Hydroxylamine HCl, 0.35 *M*. (b) NaOH 1.5 *M*. (c) Alkaline hydroxylamine. This solution is prepared just before using by mixing equal volumes of 0.35 *M* hydroxylamine HCl and 1.5 *M* NaOH. (d) Perchloric acid, 0.75 *M*. (e) Ferric–perchloric acid solution is prepared by dissolving 1.9 g of $FeCl_3 \cdot 6H_2O$ in 5 ml of concentrated HCl. To this solution is added 5 ml of 70 % perchloric acid. It is then evaporated almost to dryness and diluted to 100 ml with H_2O. The acidity of this solution is between 0.55 and 0.65 *M*. The ferric–perchloric acid solution also can be conveniently prepared by dissolving the equivalent amount of $Fe(ClO_4)_3 \cdot 6\ H_2O$* in 0.4 *N* perchloric acid.

The reagent for the deacetylation is prepared by absorbing dry HCl into refrigerated absolute methanol. An aliquot of the HCl–alcohol solution is titrated with standard alkali and a 2 *M* solution is prepared by dilution. This solution is stable for a few months in the cold if protected from moisture.

The 0.35 *M* hydroxylamine hydrochloride and ferric perchloric solutions are stable up to one month in the cold.

Procedure

An amount of the compound corresponding to 1.0 to 10 μmoles of acetyl is placed in a 70 × 7 mm pyrex tube. This solution is evaporated to dryness in a vacuum desiccator over anhydrous $CaCl_2$ by use of an oil pump. To the dried residue is added 0.5 ml of 2 *N* HCl–methanol solution. The acid methanol solution is cooled for a few seconds with a mixture of solid CO_2–methanol and the tube is rapidly sealed with an oxygen torch. The tube is heated at 100° in a water bath for 4 h, cooled, and opened close to the end of the sealed tip.

Distillation

The deacetylation tube is carefully placed in the bottom of chamber A of the

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[*] The G. Frederick Smith Chemical Co., Columbus, Ohio.

distillation apparatus, which is maintained in an upright position. Suction tube (C) is cooled to the level of the sidearm with a solid CO_2–methanol mixture during the entire distillation. After evacuation (30 to 40 mm Hg), chamber A is submerged to a depth of within 10 mm of the sidearm in a water bath at 35 to 45°. Distillation under these conditions proceeds smoothly. After all the acid alcohol solution is distilled and collected in the suction tube (C), the polyethylene cap is removed from chamber A and 0.5 ml of absolute methanol is carefully added to the deacetylation tube and distillation is repeated. This complete operation requires 20 min.

Determination of methyl acetate

After complete distillation, tube C is removed and placed in a water bath at 20–25°. After 2 min 1.0 ml of H_2O is added, followed by 2.0 ml of alkaline hydroxylamine solution. The tube is shaken and allowed to stand for 10 min. 2 ml of 0.75 *M* perchloric acid is added, the mixture is shaken, and 1.0 ml of ferric perchloric acid solution is added.

The color is read in 5 to 10 min at 520 mμ in the Coleman Junior Spectrophotometer. (For a large number of samples it has been found convenient to keep the distillates in a freezer after stoppering and covering the sidearms with parafilm until they are to be analyzed.) The quantity of methyl acetate is estimated by comparison with ethyl acetate standards.

Preparation of the standard curve

The standard solution is 0.005 *M* ethyl acetate in methanol–H_2O (50:50, v/v). Suitable aliquots of this standard are diluted to a volume of 2.0 ml with methanol–H_2O (50:50, v/v) to yield amounts varying from 1 to 10 μmoles. An HCl–methanol blank is used.

RESULTS

Factors affecting deacetylation

HCl–methanol was chosen as a deacetylation agent. Advantages of this reagent are the rapid methanolysis of acetyl groups and the ease of removal of HCl. During methanolysis, methyl chloride is formed, but this compound does not interfere with the colorimetric analysis.

Table I represents the results of experiments designed to evaluate the extent of reaction of HCl with methanol. Several concentrations of HCl in absolute methanol were heated at 100° in sealed tubes for varying lengths of time. This table shows that a rapid drop of acidity occurs during the first minutes of methanolysis.

Fig. 2 indicates the effect of HCl concentration on deacetylation. The results obtained with several compounds indicate that the maximum deacetylation is obtained with 2 *N* HCl–methanol. In the case of S-tribromo N-acetanilide, a compound in which the N-acetyl group is difficult to remove, the determination of acetyl content was accomplished by the use of 3.5 *N* HCl–methanol.

Fig. 3 illustrates the rate of deacetylation of several compounds with 2 *N* HCl–methanol. The results indicate that with these compounds, complete deacetylation is accomplished in less than 4 h.

It should be emphasized that o-acetyl groups are also liberated; consequently,

TABLE I

RATE OF CHANGE OF THE ACID MOLARITY

The acid molarity was determined by alkali titration using phenolphthalein as indicator

Time (h)	*HCl–methanol Molarity*				
0	3.67	2.76	2.08	1.50	1.00
0.25	1.16	0.94	0.75	0.50	0.43
0.50	0.71	0.63	0.48	0.35	0.28
1.0	0.40	0.35	0.28	0.20	0.15
2.0	—	0.24	0.18	0.10	0.06
5.0	—	0.08	0.06	0.05	0.04

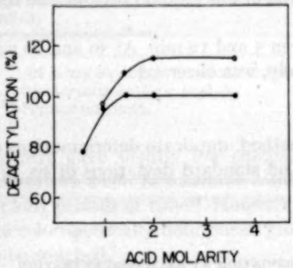

Fig. 2. Percent deacetylation of hyaluronic acid (○), and N-acetylglucosamine (●), in 4 h at 100° in sealed tubes at different acid molarities. Percent deacetylation of HA calculated on the basis of the colorimetric analysis for glucosamine[28]. Per cent deacetylation of N-acetylglucosamine calculated on the basis of N-acetylglucosamine analysis[25].

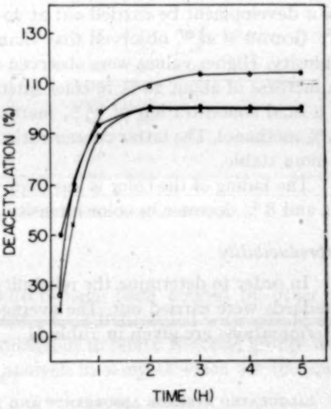

Fig. 3. Percent of deacetylation of N-acetylglucosamine (●), N-acetylglycine (×), and hyaluronic acid (○), versus time using 2 *N* HCl–methanol at 100° in sealed tubes. Percent deacetylation was calculated on the basis of colorimetrically determined hexosamine content[28]. Percent deacetylation of N-acetylglycine calculated on the basis of nitrogen determined colorimetrically by a micro-Kjeldahl procedure.

under the conditions of this method differentiation between these two groups is not possible.

Color development

The usefulness of the qualitative "spot test" first described by FEIGL[4, 5] has been established in the quantitative colorimetric estimation of carboxylic esters[6–10]. Similar reactions have been found for anhydrides[5, 11], nitrites[12], amides[13, 14], and lactones[15, 16]. Colorimetric hydroxamic acid determinations have been described for acetylated pectic acid[17] and o-acetylated proteins[18, 19].

The reagents used in this determination are essentially the alkaline hydroxylamine reagent of HESTRIN[9] and the HILL ferric perchloric acid solution[7, 16] suitably modified for this purpose.

Previous reports have indicated that hydroxamic acid formation occurs most rapidly in strongly alkaline solutions[9, 10]. However, since an acid solution is necessary for color development it is important that sufficient perchloric acid be added. A sharp decrease in the color intensity was observed when the molarity of the added $HClO_4$ was below 0.6 *M*. No changes in color intensity were obtained with $HClO_4$ solution up to 0.85 *M*.

A study has been carried out of certain other variables which influence this method. The effect of temperature and time on the formation of the hydroxamic acid in alkaline solution has been studied[16]. As previously reported[15], variations in temperature between 20° and 30° have little effect on color intensity; however, at —15° there is a marked increase in color intensity. It is, therefore, important that color development be carried out at 20–30°.

GODDU *et al.*[20], observed that changes of solvent result in change of absorption intensity. Higher values were observed when H_2O was replaced by alcohol. We found an increase of about 20 % in color intensity when color development was carried out in a final concentration of 75 % methanol rather than in the final concentration of 15 % methanol. The latter concentration is preferred in this method because the color is more stable.

The fading of the color is less than 1 % between 5 and 10 min. At 30 and 60 min, a 4 and 8 % decrease in color intensity, respectively, was observed.

Reproducibility

In order to determine the reliability of the method, duplicate determinations on standards were carried out. The average values and standard deviations of six such determinations are given in Table II.

TABLE II

CALCULATED AVERAGE ABSORBANCY AND STANDARD DEVIATION AT EACH CONCENTRATION

Concentration µmoles/7 ml	*Average absorbancy*	*Standard deviation*
2	0.281	0.007
4	0.570	0.012
6	0.837	0.012
8	1.100	0.017
10	1.375	0.016

Analyses

Although this method was devised primarily to determine quantitatively the N-acetyl groups in mucopolysaccharides, its usefulness is not limited to these compounds. Good results have also been obtained for N-acetyl amino acids and N-acetylated aromatic amines.

Table III shows the results obtained with several N-acetylated compounds. Some of these results are correlated with the chromic acid oxidation method of WIESENBERGER[29].

It is observed that in both methods the acetyl content of mucopolysaccharides is higher than the colorimetrically determined hexosamine content. This is due to the fact that the colorimetric method which was used to determine hexosamines con-

TABLE III

ANALYTICAL RESULTS*

Explanation of the letters in the Table: [a] 14-h hydrolysis with 4 *N* HCl[28] at 100°, without using Dowex 50 H+. [b] Micro-Kjeldahl procedure. [c] As obtained from Dr. A. WINTERSTEIN. [d] Purity of N-acetyl amino acids and aromatic amines were checked by their melting points. [e] Determination of N-acetyl content by using 3.5 *N* HCl–MeOH. [f] Hexosamine ratio determined by N-acetylglucosamine analysis[25].

	Hexosamine[a]	*Glucuronic acid*[21]	*Nitrogen*[b]	*N-acetyl (colorimetric method)*				*N-acetyl (chromic acid method*[27]*)*
CSA** from cartilage[22]	1.0	1.13		1.11	1.09	1.10		1.10
CSA from cartilage[22]	1.0	1.12		1.16	1.16	1.15		1.12
Umbilical cord HA*** [23]	1.0	1.12		1.10	1.11	1.12	1.10	1.13
Streptococcus HA[23]	1.0	1.13		1.16	1.15	1.16	1.15	1.10
CSA-B[24]	1.0			1.22	1.20	1.24	1.24	1.20
CSA-B [c]	1.0			1.26	1.26	1.28		
N-acetylglucosamine[25]	1.0[f]			1.01	1.00	1.02		0.99
N-acetyltryptophan [d]			1.0	1.05	1.02			
N-acetyl leucine			1.0	0.98	0.97			
N-acetyl phenylalanine			1.0	1.01	1.00			
N-acetyl glycine			1.0	1.02	1.00			
Acetanilide			1.0	1.06	1.04			
S-tribromoacetanilide[26] [e]			1.0	0.92	0.94			

* All analyses are given as molar ratios with respect to hexosamine or nitrogen.

** Chondroitin sulfuric acid-A.

*** Hyaluronic acid.

sistently gave slightly low results. Similar effects have been noticed by other investigators when hexosamine containing samples were hydrolyzed with strong acid for long periods of time[28]. However, it is advantageous to relate N-acetyl groups with the colorimetrically determined glucosamine analysis in samples which are too small to be weighed.

Compounds containing no acetyl groups, such as glycogen, heparin, glucosamine HCl, glucuronolactone and glycine gave the same color intensity as the blank. Samples containing small amounts of salts of inorganic volatile acids do not affect the N-acetyl group analysis. Salts of volatile organic acids interfere with this method.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. J. A. CIFONELLI for furnishing us with several mucopolysaccharides used in this study. The valuable technical assistance of Mr. J. E. WALKER is gratefully acknowledged.

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THE EFFECT OF OXYGEN UPON THE MICRO DETERMINATION OF HISTIDINE WITH THE AID OF THE PAULY REACTION

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SUMMARY

The colour obtained upon the addition of diazosulphanilic acid to histidine in alkaline medium (Pauly reaction) is bleached rather suddenly after a certain lag. This lag is shorter the more oxygen is present in the alkaline reaction medium.

A method of determining histidine on a micro scale, consisting of an improved Pauly reaction after paperchromatographic separation, is described in detail.

INTRODUCTION

Although accurate chromatographic methods for determining the amino acid content of protein hydrolysates are available, methods requiring but simple equipment are still useful, especially where only a limited number of amino acids need to be determined. The determination of histidine by a combination of paper chromatography and Pauly reaction, as described by Fraenkel-Conrat[1], is an example of such a method.

The present paper deals with a complication that may arise with the latter method and gives directions to avoid it.

In the Pauly reaction the red compound resulting from the reaction of histidine and diazo sulphanilic acid is measured photometrically. First sulphanilic acid and $NaNO_2$ are added to the histidine solution to be tested, whereupon the solution is made alkaline with soda solution. A red colour is then developed. Possibly because of the known instability of the colour, FRAENKEL-CONRAT[1] recommends that the extinction be read 5 min after the addition of the soda. This precaution, however, besides being rather impractical when several readings are to be made simultaneously was found by the present author to be insufficient for reproducible results owing to variability in the rate of bleaching. Individual determinations sometimes deviated as much as 50 % from the mean value.

A search for the cause of the instability of the dye revealed that the presence of oxygen after the addition of soda is one of the factors involved in bleaching. This is illustrated in Fig. 1. A reasonably stable colour is produced if the Na_2CO_3 solution is degassed *in vacuo* shortly before mixing with the diazo solution (Fig. 1 A). The latter solution is already oxygen-free due to the great excess of HNO_2 (15-fold) over the sulphanilic acid as usually recommended[2]. The soda and diazo solutions were mixed by gentle swirling.

A sudden decrease of extinction is observed if the soda solution is not degassed and mixing is carried out by gentle swirling (Fig. 1 B). The colour is even less stable if the solutions are mixed by shaking (Fig. 1 C). A very fleeting colour is observed if a current of oxygen is passed through the diazo mixture for 1 min before and 15 sec after the addition of soda (Fig. 1 D).

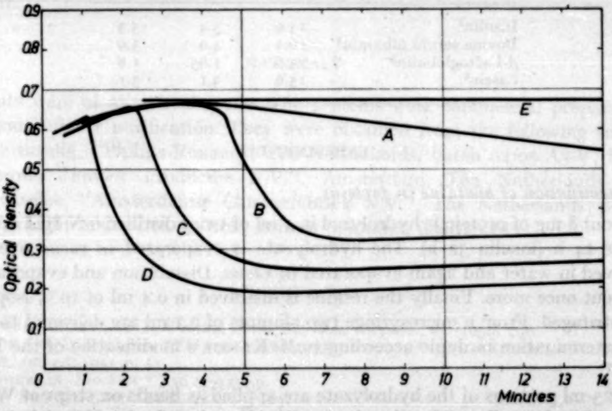

Fig. 1. Optical density of the diazo dye of histidine as a function of time. For explanation see text.

The form of the curves is very remarkable and suggests an autocatalytic reaction. Matters, however, are not quite as simple as that, since the addition of 10 % of a bleached reaction mixture to a fresh reaction mixture immediately after the addition of the non-degassed soda solution does not decrease the stability of the colour. This would have been the case if the bleaching reaction was catalyzed by the products of the bleached dye. An explanation must await further experimental data.

Curves B and C of Fig. 1 demonstrate in particular how variable the results can be if the diazo and soda solutions are inadvertently mixed.

However, even if an oxygen-free soda solution is used, the colour is not entirely stable (Fig. 1 A). Complete stability could be obtained by the addition of ethanol as recommended by McPherson[3] (Fig. 1 E). Methanol and dioxane proved to have the same effect, but acetone cannot be used because it produces a strong red colour itself.

The method of McPherson has the additional advantage that, instead of 5 % Na_2CO_3, one adds only 0.25 volume 20 % Na_2CO_3, which reduces the amount of oxygen to less than 25 %, gases being less soluble in concentrated salt solutions than in diluted ones. The data of Fig. 1 indicate that it is advisable to stir gently when the soda and diazo solutions are mixed and to add alcohol after 2 min. These precautions lead to the directions given in the experimental part.

The results of the determination of histidine in a few proteins of known amino acid content by the present method are presented in Table I. Each determination was carried out in 4-fold and individual determinations deviated by no more than 2 % from the mean value. The values obtained compare favourably with those reported in the literature.

TABLE I

HISTIDINE CONTENT OF VARIOUS PROTEINS

Protein	*N content assumed* %	*Histidine content g/100 g protein*	
		calculated	*found*
Insulin[4]	15.9	5.4	5.3
Bovine serum albumin[5]	16.1	4.0	3.9
β-Lactoglobulin[5]	15.6	1.65	1.8
Casein[6]	15.6	3.1	3.1

EXPERIMENTAL

The determination of histidine in proteins

About 8 mg of protein is hydrolyzed in 1 ml of twice distilled 6 *N* HCl *in vacuo* at 110° for 24 h (insulin 32 h). The hydrolyzate is evaporated *in vacuo* over KOH, redissolved in water and again evaporated *in vacuo*. Dissolution and evaporation are carried out once more. Finally the residue is dissolved in 0.4 ml of 10 % isopropanol and centrifuged. From a microsyringe two aliquots of 0.1 ml are delivered to be used for *N* determination in duplo according to McKenzie's modification of the Kjeldahl method[7].

0.025-ml portions of the hydrolyzate are applied as bands on strips of Whatman paper 3 MM (width 2 cm, length 35 cm), 5 cm from the bottom. Ten of these strips, 5 strips containing the histidine standard (1 mg of histidinehydrochloride · 1 H_2O/ml) and 5 strips containing the unknown, are developed for 22 h by the ascending-descending method of Block[8], using 80 % *n*-butanol as a solvent.

The chromatograms are dried and two of them (one standard and one unknown) are stained with a diazo spray. Using these chromatograms as guides, strips 2.5 cm long, containing the histidine bands, are cut from the other eight chromatograms. From the same chromatograms blank strips of 2.5 cm length are cut out from below the point of application. Each strip is tightly folded zigzagwise in two directions and introduced into a testtube (length 10 cm, width 1.1 cm), 2.5 ml of water, containing 0.25 % ethylene diamine tetraacetate is added. The cutting now partly unfolds such that it does not stick to the walls of the vessel and such that water can circulate freely. The tube is stoppered and rotated slowly head over heels for 1 h.

2 ml of the extract are pipetted into a small erlenmeyer, after which 0.2 ml of 1 % sulphanilic acid in 1.4 *N* HCl and 0.2 ml of 5 % $NaNO_2$ are added. After 10 min 0.6 ml of 20 % Na_2CO_3 is added whilst swirling gently. Exactly 2 min later 2 ml of 75 % alcohol is added and mixed by swirling without special caution. The solution is brought into a 1 cm cuvet and the extinction is read at 530 mμ.

A standard, an unknown and the two corresponding blanks are treated simultaneously. Blank strips yield an optical density of about 0.06. The blanks of the standard and of the unknown should not differ by more than 0.01 in optical density. This can be attained if the filter-paper is of good quality and the strips are handled carefully, preferably wearing rubber gloves.

Experimental conditions of Fig. 1.

Curves A–D of Fig. 1 were obtained at 20–22° under similar conditions, using 2.6 ml of 5 % Na_2CO_3 instead of 0.6 ml of 20 % Na_2CO_3 and 2.0 ml of alcohol. The concentration of histidine after the addition of all reagents was $5 \cdot 10^{-5}$ *M*. All curves except D were measured at intervals of 30 sec. For curve D the reactions were carried out directly in the cuvet and the extinctions were measured with the aid of a recording instrument.

MATERIALS

All reagents were of analytical grade. The proteins were commercial preparations, used without further purification. They were obtained from the following sources: crystalline insulin, "Philips-Roxane", The Netherlands, batch 04/05 A5-V; bovine serumalbumin, "Poviet Producten N.V.", Amsterdam, The Netherlands batch 1954-36; caseine, "Amsterdamse Chininefabriek N.V.", The Netherlands; β-lactoglobulin, L. Light & Co. Ltd.", Great Britain (no batch mentioned).

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THE OXIDATION OF L-AMINO ACIDS BY MITOCHONDRIA FROM RAT LIVER

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SUMMARY

The oxidation of L-alanine by actively phosphorylating mitochondria from rat liver has been shown. L-Alanine alone was oxidised very slowly, but, in the presence of T.C.A. intermediates and L-glutamate, there was a marked stimulation of its oxidation. The system was very responsive to low concentrations of L-alanine and to most of the T.C.A. intermediates. Of the T.C.A. intermediates α-oxo-glutarate showed the greatest stimulation. L-Alanine and L-aspartate reduced the conversion of DL-[1-^{14}C]-glutamate to carbon-dioxide. It is concluded that the probable mechanism of oxidation is not by action of L-amino acid oxidase but via transamination to α-oxo-glutarate and subsequent oxidation of the pyruvate produced. The results give evidence for mitochondrial L-glutamic dehydrogenase and transaminase as a functional cyclic system in the deamination of amino acids and as mediators of the oxidation of their carbon skeletons.

INTRODUCTION

It is known that animals have the means to deaminate amino acids and oxidize their carbon skeletons, but apart from special cases like glutamic acid little is known about the enzymic mechanisms generally involved. From the existing evidence two such enzymic mechanisms appear to be possibilities. These are the reactions catalysed by the L-amino acid oxidase of BLANCHARD, GREEN, NOCITO AND RATNER[1] and the transdeamination reactions suggested by BRAUNSTEIN[2,3] and again by ROWSELL[4]. The former system has been studied in isolation from the cell and as such is not very active. The latter system is currently being investigated by KLYUGE[5] in dispersions of liver and by BÄSSLER AND HAMMAR[6] in cell fractions of liver.

HIRD AND ROWSELL[7] found that mitochondria from liver of the rat were capable of carrying out a general reversible transamination reaction between glutamic acid and a number of other amino acids and ROWSELL[4,8] has subsequently considerably extended these observations. Mitochondria from rat liver, kidney and heart are thus established as a site of deamination. The now accepted function of mitochondria as the site of oxidation and decarboxylation of pyruvate and α-oxo-glutarate suggested to us that other α-oxo acids might be oxidized by mitochondria with the resultant metabolism of the acyl-coenzyme A derivatives.

Abbreviation: T.C.A., Tricarbonic acid.

Accordingly we decided to investigate mitochondria as a system for the deamination and oxidation of some appropriate amino acids. The present paper is concerned chiefly with L-alanine.

MATERIALS AND METHODS

Materials

Hexokinase was either prepared by the method of BERGER, SLEIN, COLOWICK AND CORI[9] or purchased from the Sigma Chemical Co. Generally labelled L-[^{14}C]-alanine and DL-[1-^{14}C]glutamic acid were obtained from the Radiochemical Centre, Amersham, England.

Mitochondrial preparations

Mitochondria were prepared essentially by the method of ALDRIDGE[11] excepting that 0.27 M sucrose was used. The mitochondrial pellet prepared from 5 g of liver was resuspended in 0.27 M sucrose. Prepared in this manner they were able to oxidize pyruvate, octanoate, glutamate, α-oxo-glutarate and succinate and were able to carry out oxidative phosphorylation. Mitochondrial protein, estimated by the method of ALDRIDGE[11] was between 2 and 7 mg/flask.

Medium

All flasks contained 0.4 ml of mitochondrial preparation in 0.27 M sucrose together with the following substances at these final concentrations: 30 mM potassium phosphate buffer pH 7.4, 1 mM ethylenediaminetetraacetate, 13 mM KCl, 26 mM TRIS buffer pH 7.4, 2 mM ATP, 5 mM $MgCl_2$, 40 mM glucose. Glucose and 25 K-M units of hexokinase were added in 0.2 ml from the side arm. The final volume in the flask was 2.5 ml, with a final pH of 7.4. The gas phase was air and the $^{14}CO_2$ was collected in the centre well in 0.2 ml of 2 N NaOH.

Estimation of $^{14}CO_2$

The alkali containing the absorbed $^{14}CO_2$ from the centre well was washed out and diluted with a suitable amount of sodium carbonate. The carbonate was precipitated and plated on paper discs (Whatman No. 3) using the method described by ARONOFF[10]. The $Ba^{14}CO_3$ was counted at infinite thickness to 1% accuracy. Results are expressed as counts/min/5 mg of mitochondrial protein.

RESULTS

Transamination between L-alanine and L-glutamate

The method used to show reversible transamination reactions between L-alanine and L-glutamate was similar to that used by HIRD AND ROWSELL[7]. The results were similar to that observed earlier and were repeated here only to determine the existence of transaminase activity in actively phosphorylating mitochondria prepared in the present way.

The oxidation of generally labelled L-alanine by rat-liver mitochondria

Table I shows that a range of concentrations of L-alanine alone did not significantly increase the respiratory activity of mitochondria over the low level of the

TABLE I

OXIDATION OF GENERALLY LABELLED L-[^{14}C]ALANINE BY MITOCHONDRIA

Results expressed as QO_2 (μl/mg protein/h) and as $^{14}CO_2$ produced/5 mg mitochondrial protein. Incubated for 1 h under conditions described in text. Counted at infinite thickness, $3.6 \cdot 10^4$ counts/min calculated to infinite thickness added to each flask. Results corrected to a standard specific activity.

Substrate	QO_2	*Counts/min*
Control (no substrate)	6.1	—
0.5 m*M* L-alanine	6.1	270
1 m*M* L-alanine	6.1	172
5 m*M* L-alanine	5.3	245
10 m*M* L-alanine	6.4	298
20 m*M* L-alanine	5.6	380

control without any addition. This suggests that L-amino acid oxidase activity of the mitochondrial preparation is either absent or of a very low order. The percentage conversion of L-alanine to carbon-dioxide is quite small and would not be observed as an increase in oxygen consumption. From the results given later with T.C.A. intermediates it is very likely that the metabolism observed results from a stimulation by small amounts of endogenous substrates associated with the mitochondria.

Fig. 1 shows that α-oxo-glutarate is able to increase 18-fold the appearance of $^{14}CO_2$ from generally labelled L-alanine (10 m*M*) and that a maximum stimulation is reached at about 10 m*M*. Oxidation of L-alanine alone was found to be variable but was at all times less than in the presence of 0.1 m*M* α-oxo-glutarate. The system is very responsive to small changes in concentration, more than half of the total activity resulting from a concentration of α-oxo-glutarate of only 1 m*M*.

Fig. 2 shows that with the α-oxo-glutarate at about saturation level (10 m*M*) the rate of oxidation of L-alanine increases with its concentration to a maximum above 20 m*M* L-alanine. A time progress curve of this reaction at two concentrations

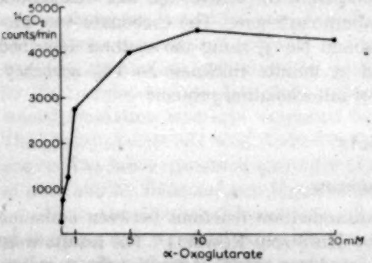

Fig. 1. The oxidation of generally labelled L-[^{14}C]alanine (10 m*M*) in the presence of varying concentrations of α-oxo-glutarate. Incubated for 1 h under conditions described in text. Counted at infinite thickness and corrected to 5 mg mitochondrial protein; $3.6 \cdot 10^4$ counts/min, calculated at infinite thickness, of generally labelled L-[^{14}C]alanine added to each flask.

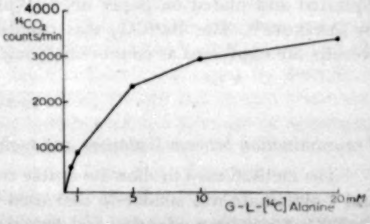

Fig. 2. The oxidation of varying concentrations of generally labelled L-[^{14}C]alanine in presence of α-oxo-glutarate (10 m*M*). Incubated for 1 h under conditions described in text. Counted at infinite thickness, $3.6 \cdot 10^4$ counts/min, calculated at infinite thickness, of generally labelled L-[^{14}C]alanine added to each flask and results calculated to standard specific activities/5 mg mitochondrial protein.

of α-oxo-glutarate can be seen in Fig. 3. This shows that the stimulation of the oxidation of alanine persists over a long period.

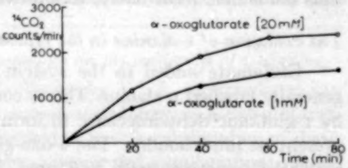

Fig. 3. Time progress curve for the oxidation of generally labelled L-[^{14}C]alanine (20 mM) in the presence of α-oxo-glutarate at 1 and 20 mM. Incubation for 1 h under conditions described in text. Counted at infinite thickness and corrected to 5 mg mitochondrial protein, 3.6·10^4 counts/min, calculated at infinite thickness, of generally labelled L-[^{14}C]alanine added to each flask.

The effect of washing the mitochondria on the system oxidising L-alanine

In order to determine whether the oxidation system was firmly bound, a mitochondrial preparation was repeatedly washed, whilst another portion of the same preparation was subjected to the same manipulations, but without washing. Table II shows the results of this experiment. There was no significant difference in activity of the much-washed preparation over the control preparation. It is concluded that the system oxidizing L-alanine is firmly bound to mitochondria and does not have, as an essential component, a contaminant from some other cell fraction.

TABLE II

THE EFFECT OF REPEATED WASHING ON THE OXIDATION OF GENERALLY LABELLED L-[^{14}C]ALANINE SYSTEM

The effect of repeated washing of mitochondria with 0.27 M sucrose on the oxidation of generally labelled L-[^{14}C]-alanine. Incubated for 1 h under conditions described in text. Counts made at infinite thickness and corrected to 5 mg mitochondrial protein, 3.6·10^4 counts/min, calculated at infinite thickness, of generally labelled L-[^{14}C]alanine added to each flask.

	Mitochondria	
	Twice washed Counts/min	*Six times washed Counts/min*
10 mM generally labelled L-[^{14}C]alanine	160	215
10 mM generally labelled L-[^{14}C]alanine + 1 mM α-Oxo-glutarate	2380	2655
10 mM generally labelled L-[^{14}C]alanine + 5 mM α-Oxo-glutarate	4960	4770
10 mM generally labelled L-[^{14}C]alanine + 10 mM α-Oxo-glutarate	5145	4900

The oxidation of L-alanine in the presence of pyruvate

Low concentrations of pyruvate added to the system result in a considerable stimulation of $^{14}CO_2$ production from generally labelled L-alanine. Fig. 4 shows the nature of the stimulation over a short time interval (10 min), as it is related to the concentration of pyruvate. The inference which is drawn from this result is that transamination allows an exchange of amino groups between the labelled L-alanine

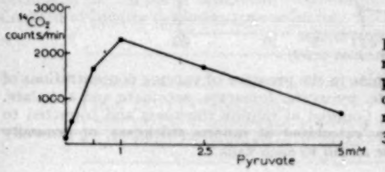

Fig. 4. Oxidation of generally labelled L-[^{14}C]alanine in the presence of varying concentrations of pyruvate. Incubation for 10 min under conditions described in text. Counted at infinite thickness and calculated to 5 mg mitochondrial protein. 3.6·10^4 counts/min, calculated at infinite thickness, added to each flask.

and the non-labelled pyruvate. The graphs shows that with concentrations of pyruvate greater than 1 mM there is an abrupt fall in the amount of $^{14}CO_2$ which is formed. This fall is due, most likely, to dilution with unlabelled pyruvate.

The oxidation of L*-alanine in the presence of other metabolites related to the T.C.A. cycle*

Glutamate added to the system also stimulates the formation of $^{14}CO_2$ from generally labelled L-alanine. This is consistent with the oxidation of the L-glutamate by L-glutamic dehydrogenase to form α-oxo-glutarate; this enzyme is known to be present on mitochondria. The α-oxo-glutarate so formed is then available for transamination reactions with L-alanine. Fig. 5 shows the relationship of L-glutamate concentration to the oxidation of L-alanine. The remaining graphs in Fig. 5 show that citrate, isocitrate, succinate, fumarate and malate also stimulate the oxidation of L-alanine and that their effects differ in detail. However, none were as efficient as α-oxo-glutarate or pyruvate. These results have been obtained with mitochondria from different rats, so that precise quantitative comparisons are not possible.

With citrate, succinate and glutamate there is a fall in each case in the amount of $^{14}CO_2$ which appears in the presence of the higher concentrations of the substrates

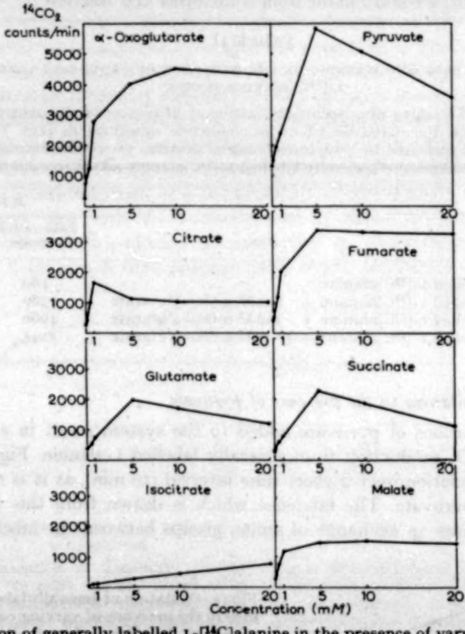

Fig. 5. Oxidation of generally labelled L-[^{14}C]alanine in the presence of varying concentrations of α-oxo-glutarate, citrate, L-glutamate, D-isocitrate, pyruvate, fumarate, succinate and L-malate. Incubated for 1 h in medium described in text. Counted at infinite thickness and corrected to 5 mg mitochondrial protein, $3.6 \cdot 10^4$ counts/min, calculated at infinite thickness, of generally labelled L-[^{14}C]alanine added to each flask.

tested. This fall can be explained in terms of dilution, as in the case of the oxidation of labelled L-alanine in the presence of unlabelled pyruvate. In the case of L-glutamate, there is the further possibility of the reverse transamination reactions due to high concentrations of glutamate.

Comparison of the effect of α-oxo-glutarate and L-malate on the oxidation of L-alanine

Stimulation of the oxidation of labelled L-alanine by T.C.A. intermediates can be attributed to the supply of oxalacetate (to accept acetyl groups) α-oxo-glutarate (to accept amino groups), from any pyruvate formed or from a combination of these. A comparison between the stimulating effect of oxalacetate and α-oxo-glutarate would therefore provide useful information about the rate-limiting step involved. However, samples of oxalacetate tested showed the presence of interfering proportions of pyruvate. Malate was then used as a substitute for oxalacetate. It can be seen from the time progress curve (Fig. 6) that, over the whole period studied, α-oxo-glutarate was the more active stimulator. Indeed α-oxo-glutarate consistently showed a greater effect than any other T.C.A. intermediate (Fig. 5). However, such an effect would also be shown if there were less dilution of labelled by unlabelled compound in the case of α-oxo-glutarate than of the other T.C.A. intermediates. In this type of experiment the superiority of α-oxo-glutarate over the other intermediate is therefore only consistent with, but not proof of, a primary role as amino-group acceptor.

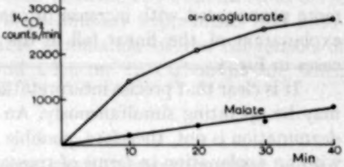

Fig. 6. Time progress curve for the oxidation of generally labelled L-[^{14}C]alanine (10 m*M*) in the presence of α-oxo-glutarate (1 m*M*) and L-malate (1 m*M*). Incubated under conditions described in text. Counts made at infinite thickness, $3.6 \cdot 10^4$ counts/min, calculated at infinite thickness, of generally labelled L-[^{14}C]alanine added to each flask.

The effect of L-alanine and L-aspartate on the decarboxylation of [1-^{14}C]α-oxo-glutarate derived from DL-[1-^{14}C]glutamate

The immediate product of the oxidation of glutamic acid in the system is α-oxo-glutarate, which may then be oxidized and decarboxylated, with carbon one of glutamate appearing as carbon-dioxide. However, in the presence of a system carrying out transamination reactions between L-alanine and L-aspartate on the one hand and α-oxo-glutarate on the other, there should be a conversion of α-oxo-glutarate back

TABLE III

THE OXIDATION OF DL-[^{14}C]GLUTAMATE IN THE PRESENCE OF L-ALANINE AND L-ASPARTATE

The effect of L-alanine, L-aspartate on the conversion of DL-[1-^{14}C]glutamate (1 m*M*) to $^{14}CO_2$. Incubated for 1 h under conditions described in text. Malonate (20 m*M*) present in each flask. Counted at infinite thickness approximately $1.5 \cdot 10^4$ counts/min, calculated at infinite thickness, added to each flask.

	Counts/min	*Inhibition*
1 m*M* DL-[1-^{14}C]glutamate	1586	—
1 m*M* DL-[1-^{14}C]glutamate + 10 m*M* L-alanine	945	41 %
1 m*M* DL-[1-^{14}C]glutamate + 10 m*M* L-aspartate	1056	29 %

to glutamate. The overall effect of such transaminating reactions would then be seen as a reduction in carbon-dioxide derived from carbon one of glutamate. Table III shows that the presence of L-alanine and L-aspartate does in fact have this effect. Malonate was added to reduce possible complications following the further metabolism of succinate to oxalacetate.

DISCUSSION

The deamination of L-alanine may proceed either by the action of L-amino acid oxidase or by transaminases, both processes forming the same products. In systems as complex as the T.C.A. cycle the factors influencing the conversion of each of the carbon atoms of L-alanine through pyruvate to carbon-dioxide are therefore not easy to assess. If the mechanism of deamination were by the action of L-amino acid oxidase, then any T.C.A. intermediate giving rise to oxalacetate would stimulate the oxidation of the pyruvate produced. Also any T.C.A. intermediate producing α-oxo-glutarate, pyruvate or oxalacetate may act as acceptors of amino groups and therefore as a means of converting L-alanine to pyruvate. Further any unlabelled T.C.A. intermediate added to the system may cause a dilution of the labelled pyruvate or more probably the labelled T.C.A. intermediates produced from pyruvate. Such dilution would be related to the extent to which the products of the metabolism of pyruvate mix with the added intermediates. This effect would be observed as an inhibition and it is likely that even the observed stimulation of the oxidation of L-alanine, by T.C.A. intermediates, contains such a component. This effect would be more pronounced with increase in concentration of the diluent and is probably the explanation of the linear fall in the rate of oxidation of L-alanine seen in certain cases in Fig. 5.

It is clear that precise interpretation of results is not possible, as the above factors may be operating simultaneously. An unequivocal description of the mechanism of deamination is not, therefore, possible. However, the following evidence is consistent with an explanation in terms of transamination.

1. The oxygen consumption in the presence of L-alanine alone has been shown to be no different from that of the control mitochondria without added substrates. BÄSSLER AND HAMMAR[6] have also shown that there is no increase in the respiratory rate of mitochondria using L-valine, L-isoleucine and L-leucine and our unpublished results with these amino acids are in agreement with theirs. ROWSELL[8] reaches a similar conclusion with respect to the oxidation of several amino acids by "particle" preparations from rat liver. These results suggest either the absence of L-amino acid oxidase or its quantitative unimportance.

2. Mitochondrial preparations from rat liver have been shown to perform reversible transamination reactions between L-alanine and L-glutamate (HIRD AND ROWSELL[7], ROWSELL[8]).

3. α-Oxo-glutarate has been found to be the most effective stimulator of the oxidation of L-alanine. Such stimulations are observed over a range of concentrations and over the complete time course investigated.

4. Pyruvate has been found to be a potent stimulator. Such a stimulation is only consistent with a mechanism by transamination.

5. L-Glutamate and the T.C.A. intermediates, all of which stimulate the oxidation of L-alanine, can be regarded as precursors of α-oxo-glutarate. KLYUGE[5], working

with homogenates of rat liver, also gives evidence for the importance of α-oxo-glutarate in the transfer of amino groups from L-alanine to citrulline.

6. When glutamate is oxidised by the mitochondrial preparation, the production of carbon-dioxide from carbon atom one of glutamate is inhibited by L-alanine and L-aspartate. This observation is consistent with transamination of the α-oxo-glutarate formed to glutamate. That the observed inhibition does not result from dilution of the α-oxo-glutarate, by metabolism of pyruvate, is suggested by the presence of this inhibitory effect when malonate is in the medium.

Mitochondria have been shown by Bässler and Hammar[6] to have increased respiration in the presence of α-oxo-butyrate, α-oxo-valerate and α-oxo-isovalerate. In experiments to be reported in a later paper we have obtained conversion of generally labelled L-[^{14}C]valine, L-isoleucine, L-leucine to $^{14}CO_2$ by mitochondria from liver and kidney tissue of the rat. Thus there is good evidence that oxidation of the α-oxo acids, produced by deamination of amino acids, can be performed by mitochondria.

Bässler and Hammar[6] also present evidence, based on oxygen consumption, that concurrent transamination and oxidation of amino acids are catalysed by mitochondria from rat liver.

From the evidence now available, it seems highly probable that the mechanism of deamination of alanine by mitochondria from rat liver is by transamination and that the amino group acceptor is α-oxo-glutarate. Such a reaction represents a cycle involving the L-glutamic dehydrogenase and transaminase(s) of mitochondria as enzymes in this cycle. This conclusion has not been yet definitely established to cover other amino acids, but it has been observed in preliminary experiments that mitochondria from rat kidney show a considerable stimulation of the conversion of generally labelled L-[^{14}C]valine, isoleucine and L-leucine to carbon-dioxide, when α-oxo-glutarate is added to the system.

NOTE ADDED IN PROOF

Still *et al.*[12], using a particulate "cyclophorase" preparation from kidney, showed a stimulation of oxygen uptake with L-alanine in the presence of α-oxo-glutarate and other TCA intermediates.

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THE EFFECT OF OXYGEN ON NITROGEN FIXATION BY *AZOTOBACTER*

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SUMMARY

Measurements of nitrogen fixation in *Azotobacter vinelandii* plotted on a LINEWEAVER-BURK plot at oxygen concentrations of 10 and 20 % indicate a competitive inhibition of nitrogen fixation by oxygen. At higher oxygen concentrations there was an additional inhibition of respiration and growth. The Michaelis constants for nitrogen fixation were 0.0107 and 0.0229 at 10 and 20 % oxygen respectively. It is suggested that oxygen and nitrogen may compete as terminal hydrogen acceptors, nitrogen fixation thus being a form of respiration.

INTRODUCTION

In an aerobic nitrogen fixing organism, such as *Azotobacter*, respiration and nitrogen fixation may be considered alternative pathways of oxidation, the hydrogen of reduced substrates being transferred ultimately in the first case to oxygen and in the second to nitrogen. If any of the same hydrogen donors are concerned in these two pathways, then, under conditions where supply of hydrogen donors is a limiting factor, we might expect to find oxygen and nitrogen competing for such donors and hence oxygen inhibiting nitrogen fixation.

Inhibition of nitrogen fixation by oxygen was first reported by MEYERHOF AND BURK[1], but this was later considered by BURK[2] to be a general inhibition of growth rather than a specific effect on nitrogen fixation, since he found a similar inhibition of growth of the organism when supplied with fixed nitrogen. PARKER[3] and TSCHAPEK AND GIAMBIAGI[4] also found inhibition of nitrogen fixation by oxygen but did not determine whether the effect was specific or due to a general depression of growth caused by inhibition of some other system.

HAMILTON AND WILSON[5] found that *Aerobacter aerogenes* could fix nitrogen under anaerobic but not under aerobic conditions, and the specific nature of the inhibition of nitrogen fixation by oxygen was shown later for this organism[6] and for a *Bacillus* of the *B. polymyxa* type[7].

This paper reports further examination of the quantitative effect of oxygen on nitrogen fixation and investigation of the nature of the inhibition by the method of LINEWEAVER AND BURK[8]. *Azotobacter vinelandii* was used as the test organism.

A preliminary note of this work has been published[9].

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MATERIALS AND METHODS

Organism

The strain of *Azotobacter vinelandii* used was kindly supplied by Dr. R. J. SWABY of the C.S.I.R.O. Division of Soils. It had several advantages for work of this kind. It was strictly aerobic and displayed no tendency to fermentation reactions at low oxygen partial pressures; it metabolised glucose or sucrose very actively; it did not alter the pH of the medium; and in suitable gas mixtures it had a generation time of about 1.5 h, even in the rigidly defined test media in which it was grown.

One serious disadvantage found was a tendency of the cells to agglutinate when grown in Warburg vessels. The clumps of cells varied in size from a few cells to several hundreds. No way was known of calculating how much oxygen was reaching the inner cells in the clumps, and it was observed that oxygen uptake usually showed a good deal of variation when agglutination was pronounced.

Attempts at obtaining "non-agglutinating" cultures by selection from the original strain met with some success, but these would revert rapidly to the agglutinating form. The same effect was found with other strains of *A. vinelandii* and *A. chroococcum* tested.

This problem was solved by centrifuging the culture lightly (about 1000 × *g* for 5 to 10 min) when it was found that the cells remaining suspended gave a culture which would grow for at least 6 h without agglutination. The oxygen consumption of such cells was found to be similar to that of the original culture but less variable.

Preparation of media

All reagents used were A.R. grade. The liquid medium was dispensed in 30–50-ml portions in 4 ounce McCartney bottles with glass caps; solid media were dispensed in 12–15-ml portions in 1 ounce McCartney bottles. These were then sterilised at 5 lbs. pressure for 30 min.

A nitrogen-free medium (M22) suitable for turbidimetric work was developed, having the following composition: sucrose 10 g, Na_2HPO_4 150 mg, K_2HPO_4 150 mg, $MgSO_4 \cdot 7H_2O$ 200 mg, $CaSO_4$ 30 mg, NaCl 100 mg, Na_2MoO_4 3 mg, $FeSO_4 \cdot 7H_2O$ 3 mg, $MnSO_4 \cdot 4H_2O$ 2 mg, $CuSO_4 \cdot 5H_2O$ 0.4 mg, $ZnSO_4 \cdot 7H_2O$ 0.8 mg, H_3BO_3 2.5 mg, ethylene diamine tetra-acetic acid Fe salt 10 mg, cysteine 3 mg, glass distilled water to 1 l. The pH was adjusted to 7.5, when necessary.

To make solid medium 2 % of Davis Bacteriological agar was added.

With the very low culture densities used in this work the rates of growth in this medium were superior to those usually reported by other workers. The phosphate content was kept low since it was found that growth was inhibited by phosphate at the levels used in BURK's[10] medium. The organism did not affect the pH of the medium under the conditions used.

Gas mixtures

Gases were mixed and stored over saturated sodium chloride solution. Argon was used as the inert diluent gas, as a product of very high purity (British Oxygen Co., 99.8 % pure) was available.

Gas concentrations are referred to throughout as percentages of one atmosphere.

Method of culture

Cultures were grown in Warburg respirometer vessels at 30°, this being a convenient method of providing a controlled atmosphere and temperature. The cultures were grown overnight with aeration, starting from an inoculum adjusted so as to ensure that the cells would be still in the logarithmic growth phase in the morning. They were then suitably diluted and aerated for 1.5 to 2 h to eliminate any lag period at the beginning of an experiment. If necessary a further slight dilution was made and 1-ml or 2-ml aliquots were transferred to Warburg flasks, of about 25 ml total gas volume with 0.1 ml of 20 % potassium hydroxide absorbed on filter paper rolls in the centre wells.

The required atmosphere was provided by thrice evacuating the flasks and refilling from a reservoir of the gas mixture[11], and all experiments were performed at atmospheric pressure.

Samples were taken at the beginning and end of the experimental period for nitrogen analyses.

Nitrogen analysis

Nitrogen analyses were carried out by an improved ultra micro Kjeldahl method[12].

Turbidimetric measurements

For preliminary estimation of the dilution required the O.D. of cultures was measured at 600 mμ in a Unicam S.P. 600 spectrophotometer in 5-mm cells. It was found that for O.D. below 0.1 there was a linear relation between amount of culture and O.D.

Optical measurements were not used as a precise measure of growth, since it was found that even in the complete absence of any source of nitrogen, free or fixed, the O.D. of a culture might increase by up to 100 % in 3 h. This may have been due to more effective use of nitrogen from parent cells by daughter cells, or to production of carbohydrate gums or other materials.

Manometric measurements

For respiration measurements the oxygen uptake was measured at 15-min intervals. The shaking rate was 107/min.

Oxygen uptake was not used as a measure of fixation as it was found that there was not a good correlation between increase in oxygen uptake and increase in fixed nitrogen[13]. The oxygen uptake however did give a useful indication of rate of growth and a means of ensuring that the right oxygen concentration was being maintained. To ensure this it was necessary to work with dilute cultures. The relation of oxygen uptake to amount of culture was found to be linear up to 400 μl/h at 20 % oxygen and up to 100 μl/h at 5 % oxygen. In order to maintain the number of cells well below the point where rate of diffusion of gas could be a limiting factor it was necessary to start from cultures with an uptake of about 80 μl/h in 20 % oxygen. Such a sample would contain 3–4 μg of nitrogen, show an O.D. of 0.02–0.03 in a 5-mm cell and give a count of about $15 \cdot 10^6$ *Azotobacter* cells by the method of GALL, STARK AND LOOSLI[14].

Glassware

With such dilute cultures in a synthetic medium traces of impurities represent a serious source of experimental variability. In particular it was found that arsenic (present in appreciable amount in the borosilicate glass used) can have a considerable effect on cultures, 0.1 μg/ml causing 20–30 % reduction in respiration rate. Variability from this or other impurities was overcome by autoclaving the vessels in 1 % nitric acid and rinsing in double glass distilled water.

RESULTS

Effect of oxygen on respiration

The effect of atmospheres varying in concentration from 5 to 100 % oxygen in argon on the oxygen uptake of *Azotobacter vinelandii* is shown in Fig. 1. There is a certain variability in the rate of respiration and the shape of the curve obtained for different subcultures, but all show a maximum at 25–30 % oxygen.

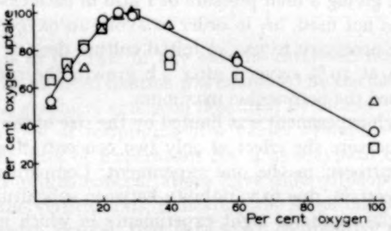


Fig. 1. Oxygen uptakes of *Azotobacter* at different concentrations of oxygen in argon expressed as per cent of the maximum uptake. Circles, triangles and squares represent results from three different subcultures.

This differs from the maximum found by Burk[2] at about 15 % oxygen. This difference could possibly be due to the different methods of preparation of the culture. Ours was grown with aeration overnight in nitrogen-free medium, so that there may have been a tendency to select oxygen resistant cells. Burk's cultures were usually 2–3 days old still cultures, diluted to give suitable oxygen consumption in the Warburg flasks, and may thus have been adapted to a lower oxygen tension. That the response to oxygen concentration is markedly affected by the atmosphere to which the culture has adapted is shown in Fig. 2. This represents the respiration at different oxygen

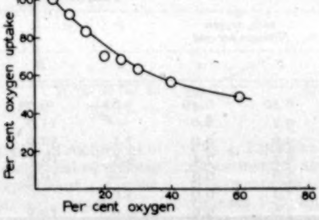


Fig. 2. Oxygen uptake at different concentrations of oxygen in argon of *Azotobacter* previously grown at 2% oxygen. Uptake as per cent of the maximum.

tensions of a culture grown on M22 agar at 2 % oxygen in nitrogen for three transfers, then overnight in M22 broth gassed with the same atmosphere, and then diluted and measured. The maximum respiration was at 5 % oxygen or less, indicating a high degree of adaptation to the atmosphere in which it grew, although the rate of respiration was only about 60 % of that of a normal culture.

The respiratory quotient was measured for oxygen concentrations of 5–100 % and found to be 0.98 ± 0.04. There was no indication of any change in the pH of the medium. Thus it would appear that, as BURK[2] concluded, *Azotobacter* completely oxidizes glucose to carbon dioxide and water.

Effect of oxygen on nitrogen fixation

The effect of oxygen concentration on nitrogen fixation was studied at oxygen concentrations 10, 20, 30 % and nitrogen 2, 4, 8, 16 %. The remainder of the atmosphere was argon giving a final pressure of 1 atm in each case.

5 % oxygen was not used, as, in order to avoid an oxygen deficiency after 3 h growth, it would be necessary to use an initial culture density too low for accurate measurement. Even at 10 % oxygen after 3 h growth the rate of oxygen uptake frequently approached the permissible maximum.

The range of each experiment was limited by the size of the Warburg bath. Thus it was possible to measure the effect of only two concentrations of oxygen at four different levels of nitrogen in the one experiment. Comparison between different experiments was uncertain, due to variability between subcultures.

Table I shows the results of eight experiments in which nitrogen fixation was measured at 10 % and 20 % oxygen.

Assuming nitrogen fixation to follow an exponential law the rate can be expressed as the velocity constant, k, defined by $\mathrm{d}N/\mathrm{d}t = kN$ where N is the fixed nitrogen concentration and t is the time (in hours). Then

$$k = \frac{1}{t} \ln \frac{\text{nitrogen at time } t}{\text{initial nitrogen}}$$

Table II shows the k values thus obtained from the data of Table I.

The value of k is thus a measure of rate of nitrogen fixation under different

TABLE I

TOTAL BACTERIAL N IN μg IN 1-ml CULTURE INITIALLY AND AFTER GROWTH IN WARBURG FLASKS
Growth for 3 h at varying oxygen and nitrogen pressures.

Expt. no.	*Initial nitrogen*	*Final nitrogen*							
		20% oxygen Nitrogen per cent				*10% oxygen Nitrogen per cent*			
		16	*8*	*4*	*2*	*16*	*8*	*4*	*2*
1	3.62	8.9	8.20	6.46	5.62	9.73	9.66	—	7.43
2	4.00	10.0	9.1	8.0	—	11.6	10.3	9.7	8.6
3	3.70	10.0	9.6	9.0	7.1	12.0	10.5	—	9.0
4	3.20	9.1	8.2	7.4	5.5	10.1	9.2	8.2	7.6
5	3.20	9.3	7.6	6.9	—	10.0	9.3	7.6	6.9
6	2.90	9.8	7.2	6.0	5.0	—	8.5	7.4	5.7
7	3.40	9.7	7.6	6.6	6.6	10.6	8.8	—	7.2
8	3.23	7.5	6.73	6.32	5.00	8.82	—	7.56	6.03

TABLE II

k VALUES FOR NITROGEN FIXATION OF CULTURES GROWN IN WARBURG FLASKS WITH VARYING OXYGEN AND NITROGEN PRESSURES

The cultures grown for 3 h.

Expt. no.	*20% oxygen Nitrogen per cent*				*10% oxygen Nitrogen per cent*			
	16	*8*	*4*	*2*	*16*	*8*	*4*	*2*
1	0.2979	0.2725	0.1927	0.1466	0.3293	0.3270	—	0.2395
2	0.3055	0.2741	0.2311	—	0.3554	0.3155	0.2956	0.2556
3	0.3316	0.3178	0.2963	0.2142	0.3923	0.3478	—	0.2963
4	0.3486	0.3140	0.2794	0.1804	0.3831	0.3524	0.3140	0.2886
5	0.3562	0.2886	0.2564	—	0.3800	0.3562	0.2886	0.2564
6	0.4061	0.3032	0.2426	0.1819	—	0.3585	0.3124	0.2257
7	0.3493	0.2679	0.2211	0.1888	0.3785	0.3170	—	0.2495
8	0.2810	0.2449	0.2242	0.1459	0.3355	—	0.2840	0.2080

conditions, analogous to the rate of any enzyme catalysed reaction. The nature of the effect of oxygen on nitrogen fixation was examined by the method of LINEWEAVER AND BURK[8] using the graphical extension described by DIXON[15].

Fig. 3 shows a plot of reciprocals of *k* values against reciprocals of nitrogen concentration for the data of Experiment No. 4 as a typical experiment. The lines obtained at the two oxygen concentrations have different slopes but essentially the same intercept on the vertical axis, indicating the same maximum rate of fixation with increasing nitrogen tension, that is to say a competitive inhibition by oxygen.

Statistical analysis of the whole group of data shows that of the two sets of eight lines at the two oxygen concentrations each can be represented by a single line. These two lines are shown in Fig. 4. The difference between their slopes is significant at the 1 % level, but there is no significant difference between the intercepts on the vertical axis. Thus we have a case of a classical competitive inhibition. The Michaelis constants obtained from the intercepts on the horizontal axis are 0.0107 at 10 % oxygen and 0.0229 at 20 % oxygen.

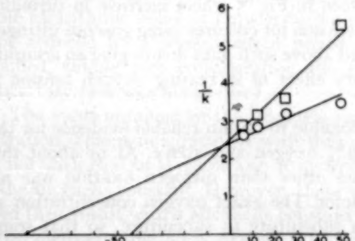


Fig. 3. LINEWEAVER-BURK plot of reciprocal of velocity constant against reciprocal of gaseous nitrogen concentration. Experiment 4. Circles: 10% oxygen. Squares: 20% oxygen.

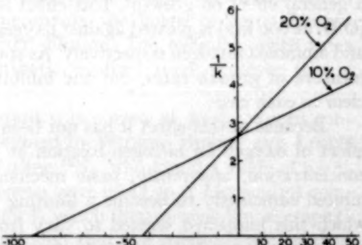


Fig. 4. LINEWEAVER plot of reciprocal of velocity constant against reciprocal of gaseous nitrogen concentration. Combined data of Experiments 1–8. Equations are: for 10% oxygen $1/k = 2.61 + 0.0279\ 1/pN_2$ for 20% oxygen $1/k = 2.68 + 0.0615\ 1/pN_2$

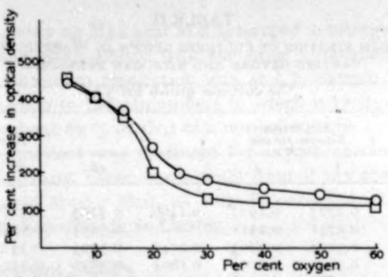
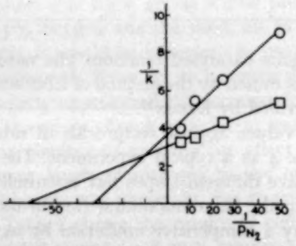


Fig. 5. Increase in O.D. of *Azotobacter* cultures grown for 4 h at different oxygen tensions. Circles, using gaseous nitrogen; squares, using ammonia as nitrogen source.



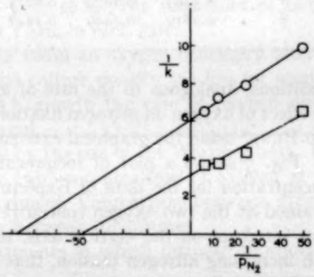


Fig. 6. **Lineweaver-Burk** plot of velocity constant against reciprocal of gaseous nitrogen concentration. Squares at 20% oxygen; circles at 30% oxygen. Culture showing almost competitive inhibition.

Fig. 7. **Lineweaver-Burk** plot of velocity constant against reciprocal of gaseous nitrogen concentration. Squares at 20% oxygen, circles at 30% oxygen. Culture not showing competitive inhibition.

Effects of higher oxygen concentrations

High oxygen tensions have, in addition to the specific effect on nitrogen fixation, a general effect on growth[2]. This effect is seen in Fig. 5 where increase in turbidity (O.D. at 600 mμ) is plotted against oxygen tension for cultures using gaseous nitrogen and ammonia nitrogen respectively. As stated above such data do not give an accurate measure of growth rates, but the inhibitory effect of increasing oxygen tension is clear in each case.

Because of this effect it has not been possible to obtain reliable evidence for the effect of oxygen on nitrogen fixation at 30% oxygen and above. At or about this concentration, apparently, some mechanism other than nitrogen fixation was inhibited sufficiently to become a limiting factor. The exact oxygen concentration at which this happened seemed to vary from subculture to subculture, so that some subcultures showed almost a competitive inhibition as between 20 and 30% oxygen (Fig. 6), others did not (Fig. 7).

DISCUSSION

The general inhibition of respiration and growth by high concentrations of oxygen

Azotobacter shows a decline with increasing oxygen tension both in respiration

(Fig. 1) and in growth, either on gaseous or fixed nitrogen (Fig. 5). This "normal" respiration curve for *Azotobacter*, obtained by BURK[2] and in the present experiments is difficult to interpret. BURK states that it is "unique", and it is certainly unlike the respiration curves of any of the organisms tested in this laboratory[16]. The respiratory activity of *Azotobacter*, however, is usually 5–10 times greater than that of an equivalent weight of other bacterial cells, and it is possible that the enzyme system is not saturated up to oxygen concentrations of 20–25 %.

The decline with higher oxygen pressures suggests the presence of an oxygen sensitive enzyme reaction in the respiratory chain. Such inhibitions have been described, for example, hydrogenase inactivation[17] or the pyruvate-oxidation inhibition found in *Micrococcus*[18].

The specific effect of oxygen on nitrogen fixation

The results presented show that oxygen inhibits nitrogen fixation. The nature of this inhibition has been studied by plotting the nitrogen fixation data according to the LINEWEAVER-BURK method, and it is demonstrated that a competitive relationship exists between oxygen and nitrogen during nitrogen fixation by *Azotobacter* (Fig. 4). This competitive relationship may be masked if the oxygen pressure is raised to a point where inhibition of growth of the organism limits the rate of fixation of nitrogen (Figs. 6 and 7).

When one attempts to measure an enzyme reaction rate which may itself be limited by the growth rate of the whole cell, and which is also affected by the respiratory activity of that cell, the results should be interpreted with some caution. In our experiments the response to nitrogen and to oxygen by *Azotobacter* varied significantly ($p < 0.05$) from experiment to experiment even though it was grown under rigidly controlled conditions. The Michaelis-Menten concept of enzyme reaction rates was derived from a consideration of an ideal enzyme–substrate system, and could only apply to nitrogen fixation under "whole cell" conditions provided that nitrogen fixation is the limiting rate reaction and that no restraints on the reaction are imposed by other requirements of growth. The calculation of k assumes that growth was exponential throughout the experiment, with no lag phase. Cultures were maintained in the logarithmic phase of growth during experiments, the danger of lag on dilution being reduced by a preincubation period after dilution. The overall growth rates obtained, taking into consideration the synthetic medium used, indicate that a satisfactory rate was maintained.

The value obtained for the Michaelis constant was 0.0229 at 20 % oxygen concentration. This is in agreement with that obtained by WILSON, BURRIS AND LIND[19] but not with that of WILSON AND ROBERTS[20] using $^{15}N_2$, who found a value of 6.6 % for the Michaelis constant. However, these workers were using 30 % oxygen but compared their result with a Michaelis constant of 2 % which they obtained by a respirometric method using 20 % oxygen. They concluded that the respirometric method gives erroneous results. While it is agreed that respirometric measurements may be misleading, WILSON AND ROBERTS did not consider the possibility that the difference they found might be due, in part, to the different oxygen concentrations used.

Nitrogen fixation and respiration

In an earlier publication[3] it was suggested that oxygen and nitrogen might compete as alternative respiratory acceptors. This hypothesis was supported by Bayliss[21] who showed that the energy changes associated with the reduction of nitrogen to ammonia are such that a substantial amount of energy could be available for work: thus, for example

$$C_6H_{12}O_6 + 4N_2(\text{gas}) + 6H_2O\,(\text{liq}) + 8H^+(\text{aq}) = 6CO_2(\text{gas}) + 8NH_4^+\,(\text{aq});\ \Delta G' = -112\ \text{kcal},$$

where $\Delta G'$ has been calculated on the basis of assumed biological concentrations of reactants. Thus it is possible that the microbe might gain useful energy from the nitrogen-fixation reaction. The biological reduction of nitrogen to ammonia, as Bayliss states, requires "no 'assistance' from any other energy-providing reaction".

The suggestion that nitrogen fixation could be regarded as a form of respiration is further supported by our results, which indicate a competitive relationship between oxygen and nitrogen. Haldane[22] considers that early forms of life were anaerobic, and that aerobic forms have developed from them. It is conceivable, too, that biological nitrogen fixation was evolved as a primitive respiratory mechanism during an anaerobic period on earth. The widespread occurrence of the nitrogen fixing ability among the anaerobic bacteria[23] lends some support to this.

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FURTHER CHEMICAL STUDIES ON THE TROPOMYOSINS OF LAMELLIBRANCH MUSCLE WITH SPECIAL REFERENCE TO *PECTEN MAXIMUS*

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SUMMARY

Two types of tropomyosin have been isolated in homogeneous, crystalline form from the adductor muscle of *Pecten maximus*. The globulin type (TM_A identical with paramyosin) has been prepared from the smooth portion, and the water-soluble variety (TM_B) from both tonic and phasic parts. Solubility and electrophoretic characteristics of the two forms are presented. The variations found in the amino acid composition of these and other molluscan TM's (both lamellibranch and cephalopod) are no greater than might be expected from species variations, but the A type as a class differs from the B in the smaller net anionic charge at physiological pH and in the replacement of some lysine groups by arginine. These two factors probably explain the different solubilities of TM_A and TM_B. The probable role of TM_A in contributing to the tonicity of smooth adductor muscles is discussed in terms of the modification of its properties in presence of ATP.

INTRODUCTION

For some years we have made a detailed chemical and physicochemical study of a globulin form of tropomyosin (TM) especially abundant in the smooth (tonic) portions of lamellibranch adductor muscle[1-5]. This form of TM was presumed by various lines of evidence to be identical with paramyosin[1,3,4], a component of certain isolated fibrils which take up electron stain in a characteristic geometrical pattern[6]. This identity was completely established when isolated crystals of TM were shown to stain in a similar way[7].

In addition to the globulin form of TM, we observed in the adductor of *Pecten* and also in the arm muscle of *Octopus* a much more soluble variety[3], crystallising in plates or sometimes in needles, and quite independently. KOMINZ, SAAD AND LAKI[8,9] also reported two types distinguished by their salting-out range in ammonium sulphate. That precipitating below 40 % saturation was called TM_A and that above, TM_B, corresponding to the globulin and water soluble types respectively. Analytical data on these various TM preparations from annelids, cephalopods, gastropods and lamellibranchs[3,8,9] indicate (a) that invertebrate TM's generally contain more arginine and less lysine, more aspartic and less glutamic than vertebrate TM; (b) that the net charge at physiological pH, whether investigated by direct electrophoresis or by analysis, is greater for the B form than the A; (c) the lys/arg ratio for the B type is higher than that for A, whilst the glu/asp ratio is lower. The diminished solubility of TM_A can partly be understood in terms of (b) and (c)[10]. The small net negative charge will not so greatly oppose aggregation, and, for reasons which are not fully understood, proteins with a high arginine content are generally globulins; moreover all proteins, including rabbit TM[11, 12], subjected to the guanidination of the lysine ε-amino group, diminish in solubility.

In the present paper we report further analyses which support the above conclusions. The new data relate particularly to the TM's of *Pecten maximus*, but *Pinna* and *Octopus* protein are also included since the previous analyses[3] have been computed in a slightly different manner. Methods for the rigorous purification of *Pecten* TM_B are given, together with electrophoretic data which illustrate the charge differences between TM_A and TM_B (see also KOMINZ, SAAD AND LAKI[8]). Physical data on TM_B of *Pecten* will be reported separately.

MATERIALS AND METHODS

Protein preparations

Tropomyosin A: The methods for *Pinna* and *Octopus* protein have already been described[3]; that for *Pecten* (from the tonic adductor) follows closely the "ethanol" method as described for *Pinna*[1, 3].

Tropomyosin B from smooth adductor: The preparation of homogenous material is quite difficult and the several steps are set out below; they combine isoelectric crystallisation, ethanol precipitation and salting-out procedures.

1. 0.5–1 kg of smooth adductors are rinsed with water and coarsely minced. The debris is washed with 3 volumes of water, squeezed out in muslin and dried in ethanol and ether. It is then stirred with 20 volumes *M* KCl, the pH adjusted to 7 with $NaHCO_3$ and allowed to extract overnight at 4°.

2. The fibre is centrifuged away and the extract dialysed against several changes of 0.2 *M* KCl, when TM_A crystallises in needles and may be purified by recrystallisation if needed.

3. The supernatant of stage 2 is adjusted to pH 5.9 with acetate buffer pH 5.4 and the precipitate discarded. The pH is now lowered to 5 with dilute acetic and the precipitate spun down. It is redissolved in 0.1 *M* KCl containing a little $NaHCO_3$ to raise the pH to 7.5 and any undissolved protein is separated and discarded.

4. To the rather turbid solution at 0°, ice-cold ethanol is added to 20 % and the precipitate discarded. Further addition to 40 % causes precipitation of a gel which is spun down and redissolved in 0.1 *M* KCl.

5. Ammonium sulphate (AS) is added at pH 7 to 30 % saturation and the precipitate spun off. To the clear supernatant more AS is added slowly to 35–40 % saturation when the TM crystallises in needles.

6. These are dissolved by dilution and dialysed against 40 % saturated AS (neutralised) when crystals appear once more. They are redissolved in water, dialysed against several changes of AS (32 g/l containing 0.02 acetate pH 5.7), and when finally equilibrated the pH is lowered to 5.3 to start crystallisation. The yield is about 1.2 % of the wet wt.

Tropomyosin B from striated adductor

Here, the minced, dried fibre obtained at stage 1 is extracted with 20 volumes *M* KCl overnight, the residue separated, and AS added to a final saturation of 30 %. The precipitate of TM_A is discarded and the supernatant is brought to 60 % saturation. The precipitate is filtered off on paper, dissolved in water, dialysed against 0.2 *M* KCl and treated as in stages 3–5. The crystals obtained by salting out are redissolved in water, and recrystallisation is effected by dialysing against AS solution (16 g/l, pH 5.4). The yield is about 0.5 % of the wet wt.

Amino acid analysis

The proteins were dialysed against water, precipitated isoelectrically, washed with water and dried in ethanol and ether. They were hydrolysed in boiling 5.8 *N* HCl for 24 h, and, after evaporating to dryness, were fractionated on Amberlite CG 120 according to the improved column procedure of MOORE, SPACKMANN AND STEIN[13, 14]. The first column of 150 cm separates all amino acids other than arg, his and lys, and the second of 15 cm separates the latter and ammonia. The yields of ser and thr were corrected for destructive losses. None of the proteins contains tryptophan (tested by the method of SPIES AND CHAMBERS[15]) or proline, and determinations of total P indicated absence of nucleic acid.

In our hands, the ammonia derived from the column, even after correction for that derived from breakdown of ser and thr, is usually greater than the true amide N liberated by the method of BAILEY[16]. For this reason direct assay of amide N has been carried out on all samples.

RESULTS

General

The crystalline forms of *Pecten* tropomyosin are shown in Fig. 1. The needles of the A type (insets 1 and 3) are quite similar to those obtained by us from oyster and *Pinna*; the B types may appear as plates (inset 4) or as needles (inset 2). These latter are of some interest since TM has never before been crystallised by a salting-out procedure.

TABLE I

ELECTROPHORETIC MOBILITIES OF PECTEN TM_A AND TM_B

Conditions	*TM_A (tonic)*	*TM_B (striated)*	*TM_B (smooth)*
I = 0.5, pH 6.8 (0.4 *M* KCl + phosphate)	2.45	4.8	5.0
I = 0.15, pH 8.0 (0.14 *M* KCl + glycylglycine)	2.75	—	7.5
I = 0.15, pH 8.0 (0.09 *M* KCl + 0.005 *M* ATP + glycylglycine)	3.3	—	7.9
I = 0.3, pH 7.0 (0.25 *M* KCl + histidine)	2.6	—	—
I = 0.3, pH 7.0 (0.2 *M* KCl + 0.005 *M* ATP + histidine)	3.1	—	—

* Ascending boundary, 10^{-5} cm^2/V-sec.

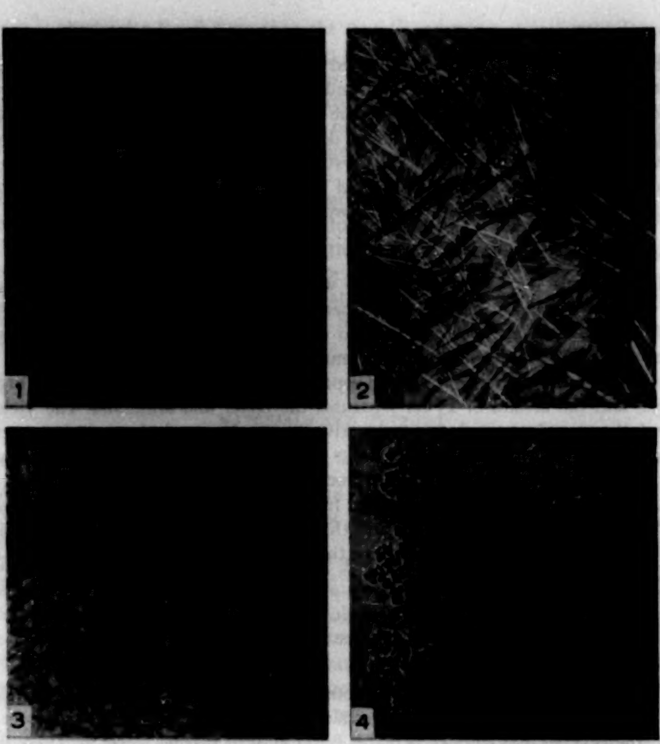

Fig. 1. Crystal forms of TM_A and TM_B from *Pecten maximus*. (1) TM_A from striated portion; (2) TM_B from smooth portion (obtained by salting-out); (3) TM_A from tonic portion; (4) TM_B from striated portion.

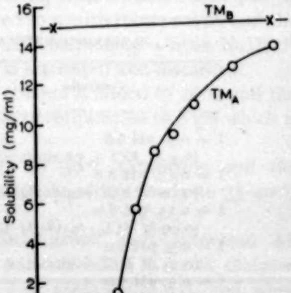

Fig. 2. Solubility of TM_A and TM_B of *Pecten maximus*. Equilibrated at 4° in 0.05 *M* TRIS, pH 7.0. Solubility determined by absorption at 278 mμ.

The electrophoretic mobilities (Perkin-Elmer apparatus, Model 38) of the various TM's are given in Table I, and agree well with values reported by KOMINZ, SAAD AND LAKI[9]. The increase of mobility in presence of ATP is commented upon later.

The marked difference in solubility of TM_A and TM_B is shown in Fig. 2. Below ionic strength 0.2, TM_A is very insoluble at pH 7, but between value of I 0.2–0.3, the salting-in curve is very steep.

Analytical

The analyses have been computed by converting the yield of α-amino N into weights of anhydro amino acid. Since the recovery of N from the columns is less than 100 % (usually about 95 %), it appears more accurate to derive the reference weight of protein, not from the N content of the sample placed on the column, but from the total weight of anhydro amino acids derived from it. The total recovery of N from the column divided by the total weight of anhydro amino acid gives the theoretical N content of the protein which may be checked against the experimental.

Table I gives the detailed analyses of TM_A from *Pecten* striated muscle and of TM_B from both striated and smooth adductors; it includes also the revised values for TM_A of whole *Pinna* adductor and of TM_A of *Octopus* arm muscle. It will be seen that the variations in the amount of any one amino acid, allowing for some divergence due to analytical error, are no more than might reasonably be expected from species variations. It seems unlikely, moreover, that TM_B from *Pecten* striated is really different from that in the tonic muscle.

TABLE II

ANALYSIS OF TROPOMYOSIN A AND B OF LAMELLIBRANCH AND CEPHALOPOD MUSCLES

Type of tropomyosin	*Weight of anhydro amino acid/100 g protein*					*Residues/10^5 g protein*				
	Pinna *A*	*Octopus* *A*	*Pecten (smooth)* *A*	*Pecten (striated)* *B*	*Pecten (smooth)* *B*	*Pinna* *A*	*Octopus* *A*	*Pecten (smooth)* *A*	*Pecten (striated)* *B*	*Pecten (smooth)* *B*
Asp	13.4	11.6	13.9	14.1	12.6	117	101	121	123	109
Thr	2.35	4.2	3.1	6.0	4.9	23.5	41	31	59	49
Ser	45	5.1	5.4	2.9	3.2	52	58	62	33	37
Glu	23.5	23.0	20.4	25.6	27.2	182	179	158	198	211
Gly	0.15	1.1	1.2	0.7	0.7	13	18.5	21	12.5	12
Ala	7.6	6.0	8.3	7.4	7.7	107	88	117	104	108
Val	3.7	3.6	4.0	3.7	3.6	37.5	36	40	37	37
Met	2.15	2.5	1.8	1.5	1.1	16.5	15	13.5	11	8.5
Ileu	3.4	4.3	4.2	2.7	3.0	30	38	38	24	27
Leu	12.4	13.1	11.9	11.0	11.2	109	116	105	97	100
Tyr	2.4	1.5	2.2	1.7	2.4	15	9	13	10.5	14.5
Phe	1.3	0.8	1.5	1.9	1.6	8.5	7.5	10	13	11
NH_3-N						(116)	(107)	(112)	(117)	(99)
Lys	8.7	9.4	9.4	12.9	12.3	68	73	73	101	96
His	0.65	1.7	1.1	nil	nil	4.5	12	8	nil	nil
Arg	13.2	12.2	11.4	7.8	8.4	84	78	73	50	54
Total	100.0	100.1	99.8	99.9	99.9	867.5	870	883.5	873	874
				Calc. N (%)		18.9	18.3	18.95	17.7	17.4
				Experimental N		18.5	18.1	—	16.5	16.3
				Average res. wt.		115	115	113	114.5	114.5

Table III compares the overall analytical characteristics of TM_A and TM_B. The amounts of total base are quite uniform throughout, in spite of the fact that the lys/arg ratio is greater in TM_B than TM_A. It seems likely, for reasons which are not fully understood, that the larger amounts of arginine contribute to the lower solubility of TM_A, an effect also enhanced by the lower net charge at physiological pH. The modification of charge is achieved by slightly raising the total dicarboxylic acid content and by slightly lowering the numbers of amidized groups *i.e.* it is a directed variation brought about by changes no larger than occur in the case of other amino acids as we pass from one species to another. Since dicarboxylic acids and their amides must be considered as quite separate entities with respect to their incorporation into protein, this method of influencing net charge is not unexpected[17].

TABLE III

ANALYTICAL CHARACTERISTICS OF TROPOMYOSIN A AND B OF LAMELLIBRANCH AND CEPHALOPOD MUSCLES

The results are expressed as % of total residues.

	Tropomyosin A				*Tropomyosin B*			
	Pinna	*Pecten*	*Venus*	*Octopus*	*Pecten*	*Venus*	*Squid*	*Rabbit*
Total acid groups (a)	34.5	32	35	32	37	37	36	34
Total base groups (b)	18	17	17	19	17	17	19	18
Amide N (c)	13.5	13.5	14.0	11.8	12.9	10.3	11.0	7.3
Net anions (a—b—c)	3.0	1.5	4.0	1.2	7.1	9.7	6.0	8.7
Non-polar	37	39	38	37	35	34	33	35
Lys/arg	0.8	1.0	0.73	0.9	1.9	1.2	1.2	2.6
Glu/asp	1.6	1.3	1.5	1.8	1.8	1.9	2.1	2.4

DISCUSSION

The analytical results discussed above for tropomyosins A and B of *Pecten maximus* confirm our own tentative conclusions on somewhat limited data[3] and those of KOMINZ, SAAD AND LAKI[9], who have presented analyses for annelids (*Lumbricus*, *Arenicola*), molluscs (*Busycon*, *Venus*) and cephalopod (*Loligo*). There seems no doubt that TM_A is widely distributed in invertebrate muscles, and in the case of molluscs at least, both A and B types coexist, with the former greatly predominating in tonic muscle. It is probable that the B form, which has now been identified in all kinds of vertebrate smooth and striated muscle, is a necessary component of the actomyosin contractile system, whilst the A form has evolved to serve an ancillary purpose. The fact that the latter constitutes over 25 % of the total protein in tonic adductors and is localised in definite fibrils where it is responsible for the "paramyosin" pattern suggests that it is linked to the special mechanical properties of these muscles, particularly to their ability to resist passive stretch and to remain contracted over very long periods. A "catch" mechanism based on the idea of a simple freezing of the muscle conflicts with the findings of LOWY and coworkers[18, 19] that intermittent excitatory volleys are always associated with tonic contraction. But there is no evidence which conflicts with the view that a "catch" implements the tetanic activity of the muscle, and we have recently investigated whether the normal extractives of muscle can influence the colloidal state of TM_A.

The results of these studies, to be reported in detail later, show that ATP at physiological concentration ($5\cdot 10^{-3}\,M$)[20,21] (a) interacts with $\mathrm{TM_A}$ to increase both solubility and electrophoretic mobility; (b) increases the plasticity of artificial tropomyosin fibres; (c) decreases the passive tension of a glycerinated tonic fibre in which the actomyosin system has been poisoned with Salyrgan or selectively denatured with ethanol. These and other types of study with model systems suggest that the great resistance to stretch in tonically contracted muscles is not entirely due to the active state of the contractile units themselves, but predominantly perhaps to the "set" of the paramyosin fibrils. If this is so, it must follow that during phasic contraction and also during relaxation, these latter must become plasticised, and the interaction of $\mathrm{TM_A}$ with ATP, if it occurs at all *in vivo*, could account for it as a purely electrostatic mechanism.

As yet, these results are no more than a basis for a suggestive hypothesis round which subsequent work may be planned. In particular, they need to be considered in relation to the fine structure of tonic muscles, and especially to the localisation of actomyosin, about which our present knowledge is extremely sketchy.

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ÉTUDE IMMUNOLOGIQUE DE LA PROTÉOLYSE DE LA CASÉINE α PAR LA PRÉSURE

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SUMMARY

An immunologic study of the proteolysis of α-casein by rennet

Immunologically, α-casein constitutes a complex antigenic system comprising at least four antigenic substances. Subjected to immunoelectrophoresis, one of these four constituents appears to be particularly sensitive to the action of rennet. The attack of rennet on this constituent is presumably concerned in the transformation of α-casein into α-paracasein during coagulation.

INTRODUCTION

La formation de paracaséine α à partir de la caséine α soumise à l'action de la présure s'effectue au cours d'une protéolyse très limitée puisque 1 à 2 % seulement de l'azote de la caséine α devient soluble dans l'acide trichloracétique[1]. D'autre part, le nombre de liaisons rompues que nous avons mesurées au cours de cette protéolyse s'est révélé particulièrement minime: 4 à 5 liaisons pour 10.000 liaisons peptidiques. Aussi l'activité de la présure ne peut être mise en évidence par les moyens ordinaires d'étude des protéolyses. Pourtant, la paracaséine α se distingue nettement par sa propriété de précipiter en présence des ions calcium et de former alors un coagulum.

Or, les techniques immunologiques, grâce à leur sensibilité, permettent de déceler des modifications très limitées du substrat si ces modifications ont porté sur le groupement antigénique de la molécule. En 1932, NICOLAS[2] a constaté des différences notables entre la paracaséine et la caséine vis à vis d'un sérum anti-caséine. Les groupements antigéniques de la caséine sont donc sensibles à l'action de la présure.

Aussi avons nous entrepris l'étude de la protéolyse de la caséine α* par la présure cristallisée en suivant son action par immunoélectrophorèse et par l'étude des surnageants après précipitation spécifique. Nous avons opéré dans des conditions analogues à celles utilisées par LAPRESLE[3,4] pour l'étude de la dégradation enzymatique de la sérum-albumine humaine.

TECHNIQUE

Immunisation

Six lapines adultes ont été immunisées selon la technique de FREUND[5] en faisant trois injections, de 10 mg environ de caséine α chacune, séparées par une semaine d'intervalle. La production maximum d'anticorps se situe environ 8 à 15 jours après la dernière injection d'antigène. Le sang a été prélevé par ponction intracardiaque*.

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* Obligeamment fournie par le Dr. T. L. McMEEKIN à qui nous adressons ici tous nos remerciements.

Electrophorèse en gélose

L'électrophorèse en gélose a été conduite selon la technique décrite par GORDON et coll.[6] et modifiée selon GRABAR et coll.[7]. Les conditions étaient les suivantes: solution de caséine α à 13 mg/ml: 0,06 ml, tampon véronal pH 8,2, force ionique 0,05 dans les bacs et 0,025 dans la gélose, tension 5 V/cm, durée 4 h. Les plaques ont été colorées à l'amidoschwarz après précipitation des protéines par l'acide acétique, et lues au photomètre.

Immunoélectrophorèse

Les analyses immunoélectrophorétiques ont été effectuées selon la méthode de GRABAR ET WILLIAMS[7]** en utilisant 2 ml de sérum anticaséine α par immunoélectrophorèse. Par ailleurs les conditions d'électrophorèse sont les mêmes que celles de l'électrophorèse en gélose.

Protéolyse

Nous avons effectué la protéolyse en milieu NaCl 0.16 M à pH = 7 à 30° en utilisant une solution de présure cristallisée préparée par C. ALAIS[8], à la concentration de 3,3 μg ou 0,13 U.P./ml du mélange caséine α et présure. La concentration en caséine α selon les expériences est soit 11 mg, soit 13 mg/ml du mélange en réaction.

La présure est inactivée par un chauffage à 80° pendant 10 sec. ou en élevant le pH à 8,2 par addition de soude N. Nous avons vérifié que ces traitements n'entraînent pas de modification du comportement immunologique ou électrophorétique de la caséine α.

Le témoin correspondant au temps zéro est obtenu en ajoutant à la caséine α une solution de présure cristallisée préalablement portée à l'ébullition.

Précipitation spécifique

Les précipitations spécifiques pour l'étude des surnageants ont été faites en ajoutant à 0,1 ml de sérum anti-caséine α, 0,1 ml d'une solution contenant des quantités croissantes de caséine α ayant subi l'action de la présure pendant un temps déterminé.

Les précipités sont conservés ensuite 1 h à 37° et 24 h à 5° puis centrifugés 20 min. à 2300 × g. Les surnageants ont été analysés selon la technique du disque (ring test), (a) avec le sérum anti-caséine α, (b) avec la caséine α non emprésurée et (c) avec la caséine α ayant subi le même temps d'action de la présure que la solution de caséine α utilisée pour la précipitation spécifique. Ces solutions de caséine α ont été employées à la concentration de 200 μg/ml.

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* Nous remercions ici M. PLOMMET qui a bien voulu se charger de ce travail.

** Nous remercions également Melle DURIEUX qui s'est chargée de la partie technique de ce travail.

RÉSULTATS

Electrophorèse en gélose (Fig. 1)

La caséine α sans présure présente un seul pic mais légèrement dissymétrique. Après 1 h d'action de la présure, ce pic se scinde en deux pics α_1 et α_2. Le pic α_2 devient plus net après 6 h et 22 h d'action de la présure. Ce résultat concorde avec celui obtenu par NITSCHMANN et coll.[9] en électrophorèse en phase liquide selon TISELIUS.

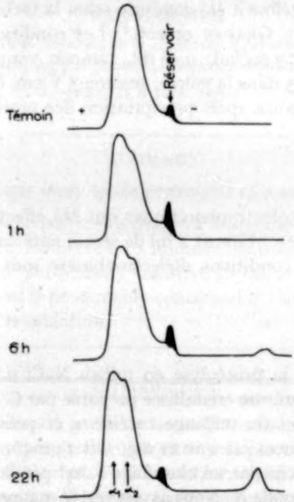


Fig. 1. Electrophorèse en gélose de la caséine α, sans présure (témoin) et après 1 h, 6 h, et 22 h d'empresurage. Présure inhibée par élévation du pH.

Il faut noter qu'au cours de l'action de la présure, il apparaît un nouveau composant migrant vers le pôle négatif. Il est déjà nettement visible sur le diagramme fait après 6 h d'action de la présure. Le diagramme d'électrophorèse de la solution de caséine α témoin n'est pas modifié si la solution est conservée à 30° pendant 22 h en présence de toluène.

Immunoélectrophorèse

La caséine à donne lieu *à* deux lignes de précipitation bien visibles (1 et 4) avec tous les sérums et à deux lignes supplémentaires très fines (2 et 3) avec certains sérums (Fig. 2). La ligne 4 se divise en deux du côté de la cathode. Ces lignes correspondent à

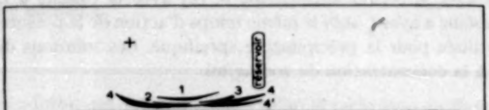


Fig. 2. Immunoélectrophorèse de la caséine α, schéma.

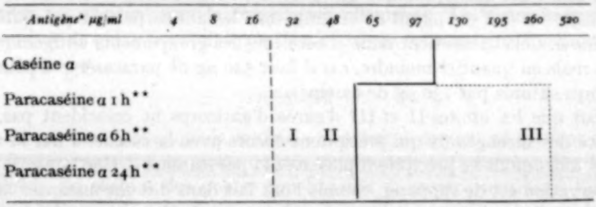
des antigènes ayant une mobilité électrophorétique très voisine dans les conditions étudiées. Au cours de l'action de la présure la ligne 1 s'estompe peu à peu pour disparaître complètement au bout d'une heure. La ligne 4 n'est pas modifiée ni dans sa forme ni dans sa position.

Etudes des surnageants après précipitation spécifique

Les résultats concernant la caséine α sont indiqués dans la première colonne horizontale (caséine α) du Tableau I. Lorsque 0,1 ml de l'anti-sérum et 0,1 ml de différentes dilutions de caséine α sont mélangés et qu'après centrifugation l'on recherche dans les surnageants la présence d'antigènes ou d'anticorps non précipités, l'on constate que dans les trois premiers surnageants (4, 8 et 16 μg de caséine α/ml de sérum) il y a seulement un excès d'anticorps. Puis dans les surnageants 32, 48, 65 et 97 μg/ml il y a toujours un excès d'anticorps mais aussi un excès d'antigènes. Enfin pour des concentrations plus élevées en caséine α, il n'y a plus d'anticorps présents dans les surnageants mais seulement de l'antigène en excès. La zone de superposition des excès d'antigènes et d'anticorps a été délimitée dans le Tableau I par la ligne en tirets I, limite d'excès d'antigènes, entre 16 et 32 μg/ml et par la ligne en trait plein II, limite d'excès d'anticorps, entre 97 et 130 μg/ml. La présence de cette zone est importante à noter. Elle indique que la caséine α ne donne pas de zone d'équivalence et qu'elle est constituée alors par un mélange d'antigènes ayant chacun un point d'équivalence différent des autres, ce qui confirme les résultats déjà obtenus en milieu gélifié.

TABLEAU I

ÉTUDE DES SURNAGEANTS APRÈS PRÉCIPITATION SPÉCIFIQUE



Antigène μg/ml*	*4*	*8*	*16*	*32*	*48*	*65*	*97*	*130*	*195*	*260*	*520*
Caséine α											
Paracaséine α 1 h**											
Paracaséine α 6 h**				I	II					III	
Paracaséine α 24 h**											

* Caséine α ou paracaséine α après 1 h, 6 h ou 24 h d'action de la présure en μg/ml de protéine ajoutés par ml de sérum anti-caséine α pour effectuer la précipitation spécifique.

** Temps d'action de la présure.

I – – – –: Limite des zones d'excès d'antigènes.

II———: Limite des zones d'excès d'anticorps lorsque les surnageants sont analysés avec l'antigène utilisé pour la précipitation spécifique.

III·········: Limite des zones d'excès d'anticorps lorsque les surnageants sont analysés avec la caséine α.

Une étude similaire a été faite avec les différentes paracaséines α, après 1 h, 6 h, et 24 h d'action de la présure, en les utilisant comme antigène pour précipiter les anticorps du sérum anti-caséine α.

La recherche des antigènes présents dans les surnageants montre que la limite d'excès d'antigènes pour les trois paracaséines α est la même que celle de la caséine α (limite I du Tableau I).

Pour rechercher les anticorps contenus dans les surnageants nous nous sommes servis de deux antigènes différents: la paracaséine α utilisée pour la précipitation spécifique et la caséine α.

Par exemple si la précipitation spécifique a été faite avec la paracaséine α après 1 h d'action de la présure (deuxième colonne horizontale du Tableau I), cette même paracaséine α est utilisée pour déceler la présence des anticorps dans les surnageants. Cette recherche permet de tracer comme pour la caséine α, une limite d'excès d'anticorps ou limite II du Tableau I. Pour des concentrations en paracaséine α plus petites que 65 µg/ml de sérum, il y a dans les surnageants un excès d'anticorps. Cette limite II est la même pour les trois paracaséines α, donc quelque soit le temps d'action de la présure entre 1 h et 24 h. La limite d'excès d'antigènes étant située entre 16 et 32 µg/ml, il existe donc toujours une zone de non équivalence, mais cette zone est plus étroite que celle de la caséine α.

La recherche des anticorps contenus dans les surnageants peut également se faire en utilisant la caséine α. Ceci donne lieu à une nouvelle limite d'excès d'anticorps, représentée dans le Tableau I par la ligne en pointillée III. Le fait d'abord que cette limite III existe, indique que pour une concentration suffisamment grande en paracaséine α (ici 520 µg/ml de sérum), tous les anticorps sont précipités par la paracaséine α. Celle-ci contient donc encore tous les groupements antigéniques de la caséine α mais en quantité moindre, car il faut 520 µg de paracaséine α pour saturer les anticorps saturés par 130 µg de caséine α.

Le fait que les limites II et III d'excès d'anticorps ne coincident pas, signifie qu'il existe des surnageants qui précipitent encore avec la caséine α par la méthode du disque, alors qu'ils ne précipitent plus avec la paracaséine α. Une façon de traduire cette observation est de supposer, comme l'ont fait dans des circonstances analogues LAPRESLE ET DURIEUX[4], que l'action enzymatique produit des antigènes "incomplètement précipitants" qui sont capables de se combiner avec les anticorps anticaséine α. Ces antigènes précipitent seulement avec les surnageants correspondant au début de la courbe de précipitation spécifique, qui sont les plus riches en anticorps.

Les résultats présentés dans le Tableau I correspondent à ceux donnés par un sérum particulier. Tous les sérums étudiés donnent des résultats analogues mais avec des variations individuelles jouant principalement sur la largeur des zones de non équivalence.

Nous avons également étudié un certain nombre de réactions croisées. Le sérum anti-caséine α réagit avec la caséine β de vache, la caséine totale de brebis et la caséine totale de chèvre, et, naturellement, avec la caséine totale de vache.

Par contre si le lait de vache donne une réaction positive par la technique du disque avec le sérum anti-caséine α, le lait de truie ne donne aucune réaction. Cela laisserait supposer que la caséine de truie aurait une spécificité immunologique complètement différente de celle de la caséine α de vache.

CONCLUSION

La caséine α à l'électrophorèse en phase liquide ou en milieu gélifié se comporte comme un produit homogène. L'étude immuno-électrophorétique révèle qu'elle est complexe et constituée par au moins quatre composants distincts. Cette composition complexe est en accord avec les travaux récents de WAUGH et coll.[10], de McMEEKIN et coll.[11], de LONG et coll.[12] montrant que la caséine α comprend un composant particulièrement sensible à l'action de la présure κ ou α_s, un autre composant en faible proportion λ et un composant résiduel α_r.

Pendant l'attaque de la présure, l'une des lignes de précipitation de la caséine α données par immunoélectrophorèse disparaît. Ceci est également en accord avec l'hypothèse que l'un des composants de la caséine α est particulièrement sensible à l'action de la présure et que de son attaque résulte l'instabilité des autres composants vis à vis des ions Ca^{++}, ce qui entraînerait la coagulation du complexe.

L'interprétation de l'étude des surnageants est très limitée en raison même de la complexité de la caséine α. Néanmoins au cours de l'action de la présure, le nombre de groupements antigéniques semble diminuer car il faut trois à quatre fois plus de paracaséine α pour saturer tous les anticorps. De plus, par l'épreuve du disque, la paracaséine α ne précipite qu'avec des surnageants contenant un taux élevé d'anticorps. Ceci est certainement en rapport avec la disparition de la ligne 1 observée en immunoélectrophorèse.

Toutes ces transformations immunologiques ont lieu au cours du temps nécessaire à la coagulation appelée phase primaire d'action de la présure et sont donc certainement en relation avec la coagulation elle-même. La phase secondaire de protéolyse générale qui se poursuit après ne modifie pas les propriétés immunologiques de la paracaséine α.

RÉSUMÉ

Au point de vue immunologique la caséine α se présente comme un système antigénique complexe comprenant au moins quatre constituants antigéniques. Par immunoélectrophorèse, un surtout des quatre constituants se montre sensible à l'action de la présure. L'attaque de ce constituant doit être en rapport avec la transformation de la caséine α en paracaséine α au cours du phénomène de la coagulation.

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STUDIES ON TUBERCULIN ACTIVE PEPTIDE

I. THE ISOLATION, CRYSTALLIZATION AND PROPERTIES OF TUBERCULIN ACTIVE PEPTIDE FROM TUBERCLE BACILLUS

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SUMMARY

A highly potent basic peptide was isolated in pure form from the bodies of tubercle bacilli. Crystallization was carried out using a methanol solution of the purified peptide. Crystals of flavianate and picrolonate of the peptide were also obtained.

The chemical and biological properties of the purified peptide are described. The tuberculin skin potency tests on human subjects indicated that the purified peptide was of a potentcy comparable to that of purified protein isolated from bacillary bodies or culture filtrate of bacilli.

INTRODUCTION

Numerous attempts have been made to isolate a biologically active protein fraction either from the culture filtrate of tubercle bacilli or from the bacillary bodies[1-6].

From these reports, the following points emerged about the tuberculin active principle.

(1) The evidence for the protein nature of the active principle has been conclusive and potent tuberculin protein molecules of many different sizes, shapes and complexities have been identified.

(2) The active protein is rather stable towards heating.

(3) Pepsin digestion of a tuberculin preparation is not able to destroy its potency completely.

These results seemed to indicate that some tuberculin activity resides even in very small protein or peptide molecules. The present study deals with the extraction, purification and crystallization of a tuberculin active peptide and some of its chemical and biological properties.

EXPERIMENTAL

Purification and crystallization of tuberculin active peptide from tubercle bacilli

Tubercle bacilli of human strain, H37Rv, were grown on Sauton's synthetic medium for seven to ten weeks and sterilized by heating at 100° for 30 min. The bacterial cells were filtered off and washed repeatedly with distilled water. After being washed several times with cold acetone, the bacilli were dried *in vacuo* and stored in a desiccator as an acetone-dried powder. About 50 g of the dried bacilli were extracted with 10 volumes (500 ml) of 0.1 *N* HCl at 37° for 72 h with frequent stirring. The mixture was filtered through filter paper and the residue was washed 2–3 times with 100 ml of 0.1 *N* HCl. The filtrate and washings were combined and filtered through a Seitz filter to remove the bacilli. The slightly opalescent filtrate was brought to pH 7.0 by the cautious addition of 5.0 *N* NaOH. It was left standing at room temperature for 2 h or more, and the flocculent white precipitate was removed by filtration.

The clear filtrate was added to an equal volume of a saturated solution of picric acid. A yellow precipitate was obtained after the mixture was left at room temperature overnight. As much of the yellow supernatant as possible was then removed by suction and the precipitate was collected by centrifugation. The precipitate was extracted overnight in 200 ml of 3 % HCl solution of 95 % ethanol, with stirring, at room temperature. The insoluble material was removed by filtration and the filtrate was concentrated *in vacuo* to one-tenth of its original volume. The concentrate was added to 10 vol. of acetone. The resulting flocculent white precipitate was collected by centrifugation after standing for 2 h at room temperature. The precipitate was washed repeatedly with acetone and dried *in vacuo*. The yield at this stage from 50 g of acetone-dried bacilli was between 50 and 75 mg.

The dried white powder was resuspended in 50 ml of a 1 % HCl solution of 95 % methanol and extracted overnight, with vigorous stirring, at room temperature. After the insoluble material was removed by filtration, the filtrate was added to 10 vol. of acetone. The precipitate was collected by centrifugation, dried *in vacuo*, and stored as "purified tuberculin peptide". The yield at this stage was about 20 mg from 50 g of acetone-dried bacilli. The preparation at this stage of purification was used for the study of biological activity.

A 5 % solution of purified tuberculin peptide in 1 % HCl in methyl alcohol was

Fig. 1. Photomicrograph of crystals of tuberculin active peptide from *Mycobacterium tuberculosis* (60 ×).

concentrated below 30° under reduced pressure and then was added to 100 ml of dried tetrahydrofuran. Glistening, pale yellow, laminated crystals appeared. Fig. 1 shows the crystalline peptide thus prepared.

Several other successful attempts have been made to crystallize purified tuberculin peptide as follows. Twenty mg of purified peptide was dissolved in 10 ml methanol, concentrated *in vacuo*, and added to methanol solution saturated with purified peptide. Glistening, laminated crystals as described above then appeared.

Thirty mg of peptide were dissolved in 3 ml of distilled water and added to 0.5 ml of 5 % flavianic acid solution. The mixture was allowed to evaporate slowly in a desiccator. Crystals of flavianate appeared after about one week. The solution was filtered and the crystals were washed once with 0.25 % cold flavianic acid solution and three times with cold water, and lyophilized. Ten mg of crystals were obtained. Fig. 2 shows crystalline flavianate of a purified tuberculin peptide.

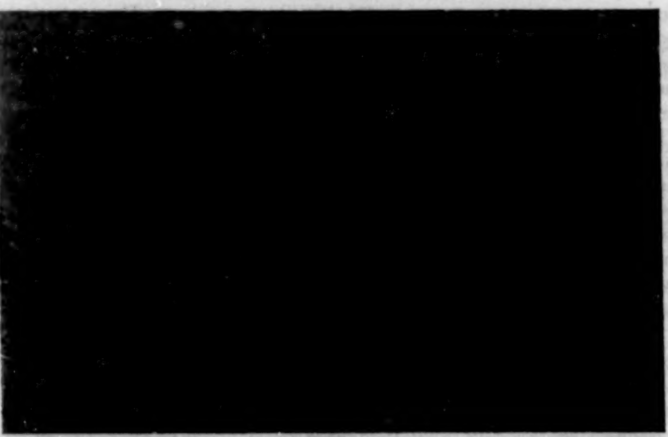

Fig. 2. Photomicrograph of crystals of tuberculin active peptide flavianate (128 ×).

Twenty mg purified peptide was dissolved in 2.5 ml distilled water and added to 0.5 ml of 1 % solution of picrolonic acid. The resulting yellow precipitate (picrolonate) was collected by filtration and washed several times with cold distilled water. The precipitated picrolonate was redissolved in 2 ml distilled water, filtered and stored in a desiccator. After 3 days yellow crystals of picrolonate of tuberculin active peptide were obtained.

CHEMICAL PROPERTIES

Chemical analysis

The nitrogen content of the purified tuberculin peptide was determined by the micro-Kjeldahl method and found to be 15.9 %. Ninhydrin, xanthoprotein, biuret, Millon's and Sakaguchi's reactions were all positive when applied to the peptide, while the Molisch, diphenylamine and orcinol tests were negative. From these results it will be concluded that there was neither sugar nor nucleic acid in the peptide.

Electrophoresis

The zone electrophoretic technique was used. In order to avoid the irreversible adsorption on cellulose, an attempt was made to use acid–ethyl alcohol-treated cellulose powder according to the method of FLODIN AND KUPKE[7]. Good quality cellulose powder (200–300 mesh) was treated by refluxing for 24 h in 10 vol. of 0.1 *N* HCl–absolute ethanol, washed several times with ethanol and distilled water, and allowed to settle until the supernatant was quite clear. The precipitated cellulose powder was washed again with ethanol and dried. For the zone electrophoresis, Kunkel's vertical type[8] apparatus was used, having a column of 2 × 40 cm.

The amount of the peptide used for electrophoretic analysis was usually 10 mg. Electrophoresis was carried out with 7 mA current at 400 V for 12 h in acetate buffer, pH 4.7 and 0.05 ionic strength. Most runs were made at room temperature; a temperature increase of 1–2° was usually observed at the end of the experiment. Elution was carried out with the same buffer; 1 ml samples were collected. Two methods, based on the ninhydrin reaction and Sakaguchi's reaction respectively, were used for the peptide determination. Independent of the reaction used purified peptide was found to consist of a single component, even after 12 h of electrophoresis. The total recovery of the peptide was 90–95 %.

Ultracentrifugal analysis

Ultracentrifugal analyses were undertaken in several concentrations of peptide (0.5–2.0 %) in acetate buffer (pH 4.7, $\mu = 0.05$) using a Spinco Model E ultracentrifuge. Schlieren photography showing the sedimentation of purified tuberculin peptide at various concentrations indicated that the boundary remains sufficiently close to the meniscus, as shown in Fig. 3. This result indicated that the peptide sediments too slowly to allow the sedimentation constant of the substance to be calculated from the movement of the maximum gradient of refractive index, and it was suggested that the molecular weight of peptide is rather small.

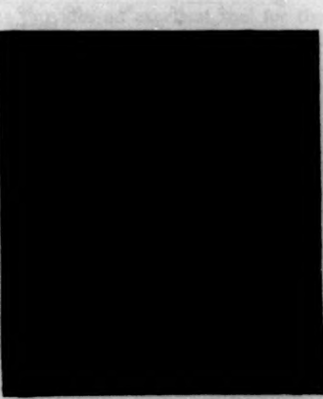

Fig. 3. Sedimentation diagram of tuberculin active peptide. Peptide concentration was 1.0 % in acetate buffer pH 4.7, ionic strength 0.05. Speed 56,100 rev./min. Exposure was taken 40 min. after reaching full speed.

Other chemical properties

The purified or crystalline peptide is easily soluble in water and rather soluble in methanol and ethanol. When diluted alkaline solution was added to peptide solution, the solution became cloudy near pH 8.5 and precipitates appeared in the vicinity of pH 9.0. Neutral solution of the peptide was heat-stable (70°, 15 min) with respect to the tuberculin activity and dialysable through cellophane membrane, indicating that the peptide is not as large a molecule as the protein component separated by SEIBERT *et al.*[6].

BIOLOGICAL PROPERTIES

Skin reaction on human subjects

Purified peptide preparation was tested intradermally on patients and students in primary and junior school. Intradermal injections of 0.1 ml amounts of proper dilutions of the peptide were made in the volar surface of the forearm approximately 2 in. below the elbow. Peptide was compared with the standard Old Tuberculin, given in the opposite arm. After 24 and 48 h the amount of induration of the reaction was measured as two diameters at right angles to each other and the mean value was described.

The comparative data of the activity of 2000-fold diluted Old Tuberculin and 0.1 μg purified peptide is shown in Figs. 4, 5 and 6. In groups of patients with

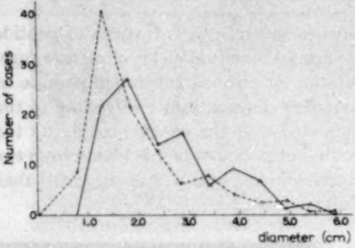

Fig. 4.

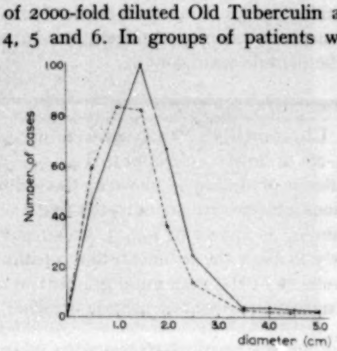

Fig. 5.

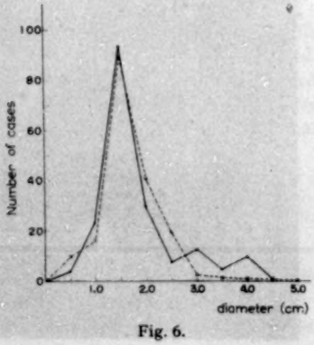

Fig. 6.

Fig. 4. Potency test of the tuberculin active peptide on tuberculous patients. Number of patients tested: 100. – – –, reaction with 0.1 ml of 2000-fold diluted old tuberculin; ——, reaction with 0.1 μg of tuberculin active peptide in 0.1 ml physiological saline solution.

Fig. 5. Potency test of tuberculin active peptide on primary school children. Number tested: 398. – – –, reaction with 0.1 ml of 2000-fold diluted old tuberculin; ——, reaction with 0.1 μg of tuberculin active peptide in 0.1 ml physiological saline solution.

Fig. 6. Potency test of the tuberculin active peptide on junior school pupils. Number tested: 191. – – –, reaction with 0.1 ml of 2000-fold diluted old tuberculin; ——, reaction with 0.1 μg of tuberculin active peptide in 0.1 ml physiological saline solution.

pulmonary tuberculosis, the reaction to 0.1 μg of the peptide was more remarkable than that to standard 2000-fold diluted Old Tuberculin. However, in groups of students in primary and junior school, the reaction with the peptide approximately equalled that with tuberculin. These results indicate that the purified peptide has a strong tuberculin activity comparable to the Old Tuberculin or the purified protein isolated from bacillary bodies and culture filtrate of bacilli.

DISCUSSION

The results in the present study show that there is a peptide in the tubercle bacilli that has a potent tuberculin activity. Several studies concerning the tuberculin active peptide were reported. TISELIUS AND GRÖNWALL[9] have found that PPD (Purified Protein Derivatives Tuberculin, Seibert) showed about one-tenth of the original tuberculin activity after digestion by pepsin and suggested that the tuberculin activity resides in small molecular peptide. TAKEYA[10] also obtained a tuberculin active principle from the digests of purified protein by trypsin, which was not precipitated by trichloracetic acid, but precipitated completely by phosphotungstic acid. KAIBARA *et al.*[11] extracted a tuberculin active substance from concentrated culture filtrate with methanol and termed it "μ". This substance was also precipitated by phosphotungstic acid and considered to be a peptide. Recently, KASUYA *et al.*[12] obtained several kinds of peptide by the hydrolysis of protein with performic acid and suggested that a peptide consisting of β-alanine, glutamic acid, glycine and cysteic acid had been proved to be the smallest unit having tuberculin activity. However, no critical study of the tuberculin potency in human and animals has so far been made with any of the preparations by these authors.

It is clear from the results of skin tests made in this study that the peptide fractions isolated from the bacillary bodies have a high potency in tuberculin reaction. Moreover, the method used here for the isolation of this peptide is simple and readily yields considerable quantities of the peptide in pure and potent form. Crystallization of a tuberculin active protein was reported by SEIBERT[13] in 1928 but did not seem to be reproducible. The method for crystallization of tuberculin active peptide described here is relatively easy and reproducible; it thus provides an excellent tool for the chemical study of the active principle of tuberculin. The amino acid composition and sequence of the purified peptide are under investigation and will be reported elsewhere.

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THE ACTIVE CENTER OF PLASMIN

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SUMMARY

Plots of the variation of pK_m and log V_{max} with pH for plasmin, determined in the absence and presence of either Ni^{++} or Ca^{++}, indicate that the active center of this enzyme is functionally identical to trypsin and that both of these enzymes differ significantly from thrombin. As with trypsin, some five groups are implicated in the active center or are affected by ES complex formation and subsequent decomposition; however, the probability exists that one or more of these groups in plasmin differ chemically from their functionally identical analogues in trypsin. Data are presented on the storage stability of plasmin and trypsin which also serve to emphasize their individuality.

INTRODUCTION

The studies reported here are a continuation of earlier research designed to identify the groups and group functions in the active centers of trypsin, thrombin and plasmin. The last report on trypsin and thrombin[1] presented data resulting from the application of the theoretical, kinetic treatment elaborated by DIXON[2] and by LAIDLER[3]. This report now extends that treatment to plasmin and makes comparison both with the previous results on thrombin and trypsin and with rechecked data on trypsin. Additional data on both the storage stability of plasmin and trypsin and as evidence of their non-identity are presented..

Abbreviation: TAME = N^{α}-*p*-toluenesulfonyl-L-arginine methyl ester.

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MATERIALS AND METHODS

Measurement of enzyme activity and the determination of K_m

The measurement of enzyme activity was performed by the null point titration procedure which has been previously described[4]. 2 ml reaction volumes were employed. 15-µl aliquots of trypsin solutions and 60-µl aliquots of plasmin solutions were used. Suitable controls were run. K_m's were determined by the method of LINEWEAVER AND BURK[5]. Five to seven points were used to establish each curve. In most cases the graphical determination of K_m leads to values for the constant which could vary by as much as $\pm 1.0 \cdot 10^{-3}$. The error on the logarithmic scale would vary with the absolute value of K_m. As most values of K_m were numerically equal to $5 \cdot 10^{-3}$ or above, a maximal variation on the logarithmic scale of ± 0.079 units can occur. For most values of K_m, the error is smaller and falls in the region of ± 0.04 logarithmic units. The conditions under which K_m was determined are as follows: 38°; initial substrate concentrations from 0.006 *M* to 0.02 *M*; initial enzyme concentration: for plasmin —2.92 Th units/ml, for trypsin —4.12 Th units/ml; above pH 6.75 veronal buffer (0.01 *M*) was used; below pH 6.75 succinate buffer (0.01 *M*) was employed. The Th unit of tryptic enzyme activity has been previously defined[4] and slightly amended[1]. This unit is as applicable to plasmin as it is to trypsin and thrombin.

Substrate and enzymes

The substrate, TAME, was a product of H. and M. Chem. Co., Santa Monica, Calif. Trypsin was a twice crystallized product and gift from Armour & Co. Plasmin was prepared from fresh beef blood as described in the next paragraph. Trypsin was stored at 0–4° in a 0.01 *M* HCl solution (pH 2.3). Plasmin was stored in the deep freeze as a lyophilized powder. Stock solutions of plasmin were prepared by dissolving suitable quantities in 0.015 *M* tris buffer at pH 7.4; these solutions were stored at 0–4°. Comments on the storage stability of these enzymes are given elsewhere in this report.

The preparation of plasmin

The method is a conglomerate of the procedures of other investigators[6,7] with significant revision from that earlier used[8], thus making a procedural recitation appropriate.

4 l of fresh beef blood was collected in 250 ml of a 1.85 % potassium oxalate plus 0.5 % oxalic acid solution. The hematocrit was centrifuged off (2,600 × *g*, cold) resulting in 1.2 l of plasma; 40 ml of 5.3 % $CaCl_2$ was added and the plasma was refrigerated overnight. However, no clot formed. Addition of 1,000 NIH units of thrombin caused clotting in 5 min. The fibrin was centrifuged off (2,600 × *g*) leaving about 800 ml of serum, which was mixed with 200 ml of 0.15 *M* tris buffer at pH 7.4 and placed overnight in the deep freeze. In the morning, the frozen solution was thawed at room temperature and centrifuged to remove undesirable material. The solution was divided into two parts and each part was shaken with 100 ml of chloroform for 1 h at room temperature. The chloroform was removed; the resulting serum adjusted to pH 5.5 with dilute sulfuric acid and refrigerated overnight. The precipitate that formed was centrifuged off (2,600 × *g*, cold) and dissolved in 100 ml of 0.015 *M*

tris buffer, pH 7.4. The protein solution thus obtained was devoid of plasmin activity. It was treated with 2 mg of trypsin for 1 h, followed by 20-fold dilution and adjustment to pH 5.5 with dilute sulfuric acid. After a night's refrigeration, a white precipitate formed. The supernatant was decanted; the residue was retrieved by centrifugation (10,500 × g, cold, 20 min) and dissolved in 50 ml of 0.015 M tris buffer at pH 7.4. This solution had no fibrinolytic activity. 20 mg of trypsin (20 mg commercial preparation = 10 mg of enzyme) was added and the solution was incubated (25°; 1.5 h), followed by 20-fold dilution, adjustment to pH 5.5 and overnight refrigeration. After decanting most of the supernatant, the plasmin precipitate was centrifuged off (2,600 × g, 25°) and redissolved in 30 ml of 0.015 M tris buffer, pH 7.4. Solution was fairly rapid and complete after a night of refrigeration. This solution had potent fibrinolytic activity; a 0.1-ml aliquot prohibited clot formation of an aliquot of fibrinogen solution which readily clotted in its absence. The plasmin solution was diluted to 600 ml, and on adjustment to pH 5.5 with 0.1 N H_2SO_4 soon became cloudy. It was placed in the refrigerator for 4 h. The precipitate was centrifuged off (2,600 × g); dissolved in 25 ml of distilled water and lyophilized for 8 h. A white, fluffy powder was obtained (0.62 g); total activity: 4,000 Th units (equals 6.75 Th units/mg of dry preparation). This value represents about 3 to 3.5 times greater activity than the previous preparation[8]. In the above procedure, the pretreatment with 2 mg of trypsin was insufficient to produce activation, but may have been instrumental in acting as a "protein and inhibitor cleansing agent" thus permitting the subsequently added trypsin to act more fully and to produce greater activation of plasminogen to plasmin.

RESULTS AND DISCUSSION

Storage stability of plasmin and trypsin

Plasmin stored as a lyophilized powder in a screw-top bottle in the deep freeze (—15°) was stable for at least two months. However, the requirement for *in vacuo* storage at this temperature stated by Jackson and Mertz[7] proved unnecessary. As dry storage of the enzyme is undesirable for its daily use, the stability of plasmin in solution was investigated. Fig. 1 contains the results which are obvious upon inspection; affirming the already known stability of trypsin[4,9] at pH's in the vicinity of 2.3. The data serve as a guide for the storage of these enzymes under conditions which will assure a relatively constant level of activity (within the bounds of experimental error) for reasonable lengths of time. In addition, the observation that plasmin is unstable at a pH where trypsin is stable (pH 2.3) while, to the contrary, being stable at a pH (7.4) where trypsin is unstable, adds another line of evidence to the six items previously listed[8] to support the conclusion that trypsin and plasmin are remarkably similar but definitely not identical enzymes. Striking emphasis of this conclusion in terms of the active center is presented in the material that follows.

The active center of plasmin

This discussion presumes a familiarity with the kinetic analyses of Dixon[2] and Laidler[3] and with the previous report on trypsin and thrombin[1].

Fig. 2 presents plots of the pK_m against pH and of the corresponding $\log_{10}V_{max}$ *vs.* pH for the reaction between TAME and plasmin. These curves are hereinafter referred to as the pK_m or the log V curves.

In those pH regions where the log V curves are horizontal straight lines, k_3 (the ES decomposition constant) is unaffected by the discontinuities in the pK_m curves and, further, these discontinuities indicate ionizations which affect or are affected by the formation of the ES complex. As the guanidino group of TAME remains cationic in the pH regions investigated here, the changes in the pK_m curves refer to dissociations on either the free enzyme or the ES complex. Further, any electrostatic combination between the substrate through its positively charged guanidino group and a negatively charged group on the enzyme would produce no alteration in total charge on ES complex formation and thus would not cause any deviation in the pK_m curve from a horizontal position. The slopes of all non-horizontal lines, shown by the dashed extensions, approximate either +1, +2, —1, or —2 values. Groups with a +1 slope can be described by the following reaction prototype on the sloped portion of the curve: $EH^+ + S \rightarrow ES + H^+$; on the other hand, those having a —1 slope yield a reaction prototype on the sloped portion of the curve as follows: $E + H^+ + S \rightarrow EH^+S$.

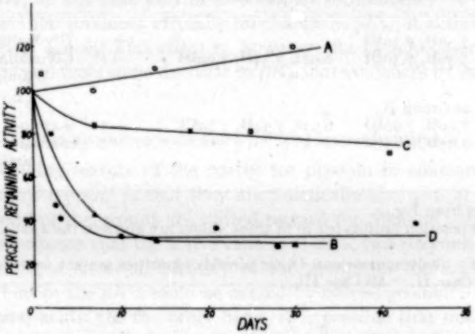

Fig. 1. Storage stability of plasmin and trypsin. Curve A, trypsin stored at pH 2.3 (HCl solution), 0–4°. Curve B, trypsin stored at pH 7.4 (0.015 *M* tris buffer), 0–4°. Curve C, plasmin stored at pH 7.4 (0.015 *M* tris buffer), 0–4°. Curve D, plasmin stored at pH 2.3 (HCl solution), 0–4°. Tests of activity conducted at 38°; pH 8.0; 0.01 *M* TAME as substrate; 0.01 *M* veronal buffer; 2-ml reaction volume.

In Fig. 2, indicated groups are designated by the capital letters drawn close to the various inflection points. To avoid confusion, Table I presents the p*K*'s, slopes and identity suggestions of the possible groups in summary form. Curves II and III, Fig. 2, are virtually identical but differ from Case I in that the p*K*'s of several of the groups are shifted to alkaline regions about 0.6 to 0.8 pH unit. A shift of similar degree for the analogous cases has been earlier found with trypsin and thrombin[1]. The inflection at 7.30 (Cases III and II) and at 6.56 (Case I) mark a point of transition in each case from +1 to —2 slope and is indicative of three groups on the free enzyme, that is, one responsible for the +1 slope (Group A) and two (Groups B and C) responsible for the —2 slope. At pH 7.60 (Case III), 7.54 (Case II) and 6.92 (Case I) the transitions from the —2 slope to a +1 slope indicate three groups on the ES complex. Waves or steps in the pK_m–pH curves indicate shifts in group p*K* values in the free enzyme from what they are in the ES complex and *vice versa*. The curves in

TABLE I

GROUPS ON PLASMIN*

Group designations	Group pK		Slope**	Possible group identities***
	On free enzyme	On ES complex		
A	6.56§, 7.30§§, 7.30§§§		+1	Glyoxaline (histidyl)
B	6.56§, 7.30§§, 7.30§§§	6.92§, 7.54§§, 7.60§§§	—1	Glyoxaline (histidyl) or α-amino
C	Same as Group B			
D	7.75§, 8.00§§, 7.96§§§	6.92§, 7.54§§, 7.60§§§	+1	α-amino
E	9.01§, 9.07§§, 9.03§§§		—1	α-amino, sulphydryl or ε-amino

* Values taken from Fig. 2.

** Examples of reaction prototypes to fit these values are given in the RESULTS and DISCUSSION section.

*** These are only likely suggestions. Other possible identities are not excluded.

§ Case I. §§ Case II. §§§ Case III.

Fig. 2 about the points discussed above can be mentally resolved into the forms and . Thus the two groups, B and C, responsible for the —2 slope on the ES complex with pK values of 7.60, 7.54 and 6.92, respectively, would then be the same as those having pK values of 7.30, 7.30 and 6.56 on the free enzyme in the same respective cases. Group D, which is responsible for the +1 slope on the ES complex at 7.60, 7.54 and 6.92 would then be the same group on the free enzyme which gives rise to pK's at 7.96, 8.00 and 7.75 in Cases III, II and I, respectively. As in the earlier case of trypsin[1], it is again important to note that according to DIXON[2], "if a substrate does not actually combine with the group in question but merely affects its ionization so that its pK is not the same in the ES complex as it is in the free enzyme, a wave will appear in the curve, etc.". Thus, Groups B, C and D are presumably not involved in ES complex formation. The additional points of inflection at pH 9.03, 9.07 and 9.01 in Cases III, II and I, respectively, represent a group, E, on the free enzyme. From the above, it appears that during ES complex formation and subsequent decomposition some five groups are either involved in the reaction; that is, are members of the active center, or their pK's are affected by the hydrolytic process. Table I contains suggestions for possible chemical identities of the various groups. It is emphasized that these are merely suggestions; other possibilities are not excluded.

The data from the log V curves indicate that Groups A, E and at least one of the three, B, C and D, have no influence on the rate of ES complex decomposition (k_3). In Case I, the +2 slope with an inflection point at 6.88 indicates that two of the three Groups, B, C and D, affect k_3. In Case II, one of these two groups exhibits virtually the same pK as in Case I, namely at 6.82; whereas the other group has its pK shifted some 0.7 pH unit to 7.52. When Ni^{++} is present (Case III), the shift observed in Case II for one of the groups is maintained and an identical inflection point to that found in the latter case is observed. However, the group with inflection points at 6.88 and 6.82 for Cases I and II, respectively, apparently loses its influence when Ni^{++} is present. It is reasonable to presume that this group interacts with Ni^{++}. This situation is virtually identical to that observed earlier in the case of trypsin[1], except that the shifting of the pK's of both groups in the presence of Ca^{++} is observed with this enzyme.

The influence of two groups of the three, B, C and D, on the rate of ES complex decomposition, k_3, is of further interest in view of the fact that these groups, as pointed out above, do not take part in ES complex formation.

Whereas Ca^{++} ion produces virtually no change in pK_m, it activates plasmin by an augmentation of the k_3. This effect is, however, not clearly observable from the log V curves. Ni^{++} produces some decrease in pK_m, but evidences its inhibition mainly by a decrease in k_3.

Comparison of the plasmin curves with those for trypsin and thrombin

The most striking feature of the curves for plasmin in comparison with those previously given for trypsin[1] is that they are practically identical in shape, but that the pK's of several of the groups are shifted toward the alkaline region. This makes reasonable the conclusion that the active centers of these two enzymes are *functionally* identical. Assuming a chemical identity of the groups in the active centers, the observed variations in the pK's could be caused by spacial geometry or the effects of neighboring amino acids. On the other hand, it is possible that one or more of the groups in one enzyme may be chemically different from the analogous groups in the other enzyme, yet functionally identical. Thus, either a glyoxaline histidyl group or an amino group can yield identical reaction prototypes and, therefore, an identical pK_m curve in the region of the group's pK, but would most probably show different pK's. Phenomenal, too, is the fact that the magnitude of the shift of approximately 0.6 pH unit for the pK's of Groups A, B and C and 0.4 pH unit for Group D observed for trypsin when either Ca^{++} or Ni^{++} are present, compared to the case of their absence[1], finds parallel in the data for plasmin, the shift being of only slightly different

Fig. 2. The variation of pK_m and the corresponding $\log_{10}V_{max}$ with pH for plasmin. Conditions and procedures are as described in the MATERIALS AND METHODS section. Case I, plasmin alone. Case II, plasmin plus 0.0066 M $CaCl_2$. Case III, plasmin plus 0.001 M $NiCl_2$.

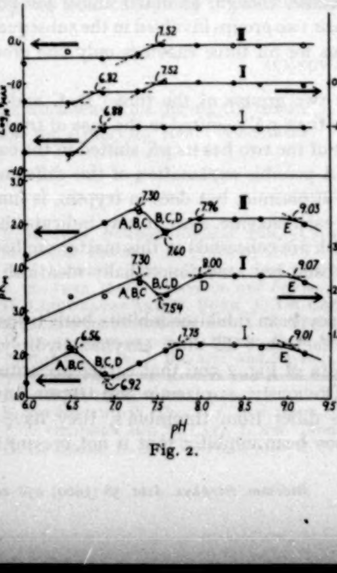

Fig. 2.

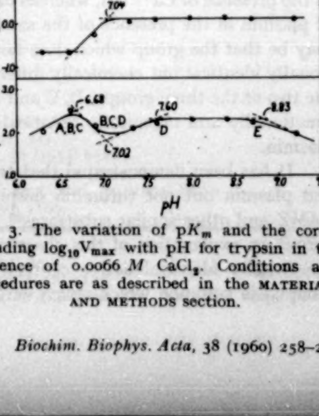

Fig. 3. The variation of pK_m and the corresponding $\log_{10}V_{max}$ with pH for trypsin in the presence of 0.0066 M $CaCl_2$. Conditions and procedures are as described in the MATERIALS AND METHODS section.

dimension (0.6 to 0.8 pH unit for Groups A, B and C and 0.2 to 0.3 pH unit for Group D). For Group E a remarkable correspondence is observed for plasmin in all three cases and the values are shifted about 0.2 unit from what they are for trypsin.

As the previous work on trypsin and thrombin involved the use of a different Model G Beckman pH meter, different standard buffer, electrodes, and other factors from those used in this study, and in view of the use of trypsin to activate plasmin, the thought was considered that perhaps the effects are really those of trypsin and that some factors in the previously used system are at variance with the same factors in the present system, thus explaining the difference between the earlier trypsin curves and the "suspect" plasmin used here (the various lines of evidence demonstrating the non-identity of trypsin and plasmin previously given[8] and as presented in an earlier paragraph in this report, notwithstanding). Therefore, the same trypsin preparation as earlier used was employed in a re-run of the case of trypsin in the presence of Ca^{++} ion. The curve that resulted, Fig. 3, is identical within experimental limits to that earlier obtained[1] (inflection points observed here at 6.68, 7.02, 7.60 and 8.83; earlier at 6.72, 7.08, 7.69 and 8.87). These data establish the validity of the earlier data on trypsin and thrombin[1] and of the present data on plasmin. Additionally, they spotlight the remarkable similarity but non-identity of plasmin and trypsin (compare Fig. 3 with Case II, Fig. 2).

For comparison with thrombin, the earlier remarks on trypsin[1] are equally applicable to plasmin. Thus, the difference in the manner by which Ca^{++} causes its activation toward thrombin as compared to trypsin is valid for thrombin as compared to plasmin. Also, Ni^{++} inhibits all three enzymes in the same general manner, apparently acting identically toward plasmin as toward trypsin. The similarity between Group D in trypsin and Group B in thrombin is likewise applicable to Group D in plasmin. As with trypsin, plasmin has two groups involved in ES complex formation (Groups A and E) and at least two involved in the subsequent hydrolysis (two of the triad, B, C and D). This situation is duplicated with thrombin except that only one group is involved in the subsequent hydrolysis[1], though, as noted above for both trypsin and plasmin, the influence of one of their two groups involved in the subsequent hydrolysis is lost when Ni^{++} is present. Thus for all three enzymes only one group appears essential for the hydrolytic step.

It has been mentioned above that the two groups of the three, B, C and D, whose p*K*'s appear on the log V curves, have their p*K*'s shifted in the case of trypsin in the presence of Ca^{++} ion, whereas only one of the two has its p*K* shifted in the case of plasmin in the presence of the same ion. A possible explanation of this difference may be that the group which does not shift in plasmin, but does in trypsin, is functionally identical but chemically different in each enzyme. Also, it may indicate that the two of the three groups, B, C and D, which are concerned in this matter are both functionally and chemically identical in trypsin, but only functionally identical in plasmin.

It has been demonstrated that trypsin soy bean inhibitor inhibits both trypsin and plasmin but not thrombin despite the fact that all three enzymes hydrolyze TAME and other similar substrates[10]. The data of Fig. 2 and that earlier presented[1] permit an explanation of this observation. Obviously, as plasmin and trypsin have functionally identical active centers, which differ from thrombin's, they have a group(s) in common which reacts with the soy bean inhibitor that is not present in thrombin. Groups B and C in plasmin and trypsin appear to have no counterpart in thrombin and may well be the group(s) involved.

The observation that plasmin and trypsin have functionally identical active centers although the group p*K*'s are different may be evidence of at least one mechanism whereby biological activity is controlled by nature. For plasmin as compared to trypsin, the p*K*'s are all shifted from 0.2 to 0.8 pH unit toward the alkaline side. Thus, at the blood pH of 7.4, much less of any molecular concentration of plasmin would be in the active form than a corresponding molecular concentration of trypsin.

The data also explain the earlier observation that over a short interval of time (5 min) plasmin and trypsin are relatively stable to acid treatment whereas thrombin is rapidly destroyed under the same conditions[8].

GLADNER AND LAKI[11] and DIXON *et al.*[12] have demonstrated the presence of the amino acid sequence, gly·asp·ser·gly, in thrombin and trypsin which they claim is part of the active centers of these enzymes. In view of the identity of the pK_m curves for plasmin and trypsin, this sequence may also be present in plasmin. Further, the pK_m curves presented both here and in the earlier work on thrombin and trypsin[1] would only have uncovered the action of the seryl hydroxyl group. However, the data on all three enzymes, while not excluding the function of such a group, do indicate that the active centers of thrombin, trypsin and plasmin are considerably more complicated than the single tetrapeptide "thread" mentioned above.

As a final conclusion, the data indicate that in terms of the active center, plasmin is functionally identical but slightly different from trypsin, while both differ to a relatively greater extent from thrombin. Although all three enzymes hydrolyze the same substrate types, the additional possession of clot catalyzing properties by thrombin may be the resultant either of a group it possesses which has no counterpart in plasmin or trypsin (Group C in thrombin)[1] or that the at least two groups in plasmin and trypsin (B and C) having no seeming counterpart in thrombin prevent these enzymes from catalyzing the clotting process.

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L'OCYTOCINE ET LA VASOPRESSINE DU MOUTON: RECONSTITUTION D'UN COMPLEXE HORMONAL ACTIF

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(Reçu le 9 juin, 1959)

SUMMARY

Oxytocine and vasopressine of sheep: reconstitution of an active complex

From sheep posterior hypophysis a complex was obtained containing 90 % of the oxytocic and vasopressic activity of the gland. The complex is an association of oxytocine and vasopressine with a protein, neurophysine. This complex was dissociated by TCA and the three components were purified by chromatography. The reconstitution of the complex from the elements was realised under specified conditions. Analogous complexes were obtained from glands of cow, horse and pig. It is possible to bring about associations between the hormones and neurophysines of different species.

INTRODUCTION

L'ocytocine et la vasopressine contractent avec une protéine de la post-hypophyse, la neurophysine, une association particulière[1]. Cette propriété a été utilisée par ACHER, LIGHT ET DU VIGNEAUD[2] dans la mise au point d'un procédé de purification rapide des deux peptides. La méthode a permis récemment d'isoler les hormones du cheval[3] du mouton[4] et de l'homme[5]. Le présent mémoire d'une part apporte des informations particulières sur la formation du complexe dans l'extrait de glandes de mouton, d'autre part précise les conditions de sa reconstitution à partir de l'ocytocine, la vasopressine et la neurophysine.

PARTIE EXPÉRIMENTALE

Techniques utilisées

Dosage de l'activité ocytocique: L'activité ocytocique a été dosée en mesurant la contraction de l'utérus de rate, selon la méthode de HOLTON[6].

Dosage de l'activité vasopressique: L'activité vasopressique a été déterminée d'après l'élévation de la pression sanguine chez le rat anesthésié selon le procédé de LANDGREBE *et al.*[7].

Chromatographie sur carboxymethylcellulose· L'échangeur utilisé possède une capacité de 0.55 mequiv./g et provient d'une cellulose Solka-Floc type 20 (Lot 1007–Brown C°, Berlin, New Hampshire, USA). La cyclisation est effectuée suivant PETERSON ET SOBER[8].

Chromatographie sur Amberlite-IRC 50 (XE-64): L'échangeur est traité suivant Hirs *et al.*[9], et tamisé sous forme de sel de sodium. La fraction passant au tamis 100 est utilisée.

Préparation du complexe actif

Le matériel de départ est une poudre acétonique de posthypophyses de mouton*. Chez cette espèce, l'hypophyse possède un lobe postérieur minuscule, représentant environ le 1/50 de la glande, et pesant environ 10 mg à l'état sec.

Le Tableau I indique, à titre de comparaison, les teneurs en activités ocytocique et vasopressique des poudres acétoniques de post-hypophyses de quatre espèces de mammifères.

TABLEAU I

ACTIVITÉS BIOLOGIQUES DES POUDRES ACÉTONIQUES POST-HYPOPHYSAIRES

Espèce	*Activité ocytocique unités/mg*	*Activité vasopressique unités/mg*
Boeuf	1.0	1.1
Porc	1.4	1.4
Cheval	0.9	0.8
Mouton	0.8	0.8

Les préparations de complexe actif de mouton ont été effectuées sur des lots de 30 g de poudre titrant 0.8 unités U.S.P. ocytocique ou vasopressique par mg. On extrait une première fois par 1 l d'acide sulfurique 0.01 *N* (20 h à 5°) puis une deuxième fois avec 500 ml d'acide. Les extraits sont réunis (1.3 l) et le pH est ajusté à 3.9 avec de l'acide sulfurique *N*. Le complexe actif est alors précipité par addition de chlorure de sodium à raison de 100 g/l (16 h à 5°). Le précipité est séparé par centrifugation, remis en suspension dans 150 ml d'eau et dialysé jusqu'à élimination totale du chlorure. Le pH est ajusté à 3.9 (solubilisation du complexe) et le volume est porté à 700 ml avec de l'eau. On ajoute alors un volume égal de tampon acétate de sodium *M* à pH 3.9. On effectue ensuite une précipitation fractionnée à l'aide de chlorure de sodium.

Lors de la préparation du complexe actif à partir des glandes de boeuf[1], de porc[10] et de cheval[3], on recueille la fraction précipitant entre 25 et 65 g de NaCl/l. Nous avons constaté qu'une précipitation importante du complexe s'effectue ici avant d'atteindre le titre de 25 g de NaCl/l et nous avons donc recueilli la fraction précipitant entre 10 et 65 g (une nuit à 5°). Le précipité est séparé, redissous dans 125 ml d'eau et après addition d'un volume égal de tampon acétate *M* pH 3.9, le complexe est à nouveau précipité par addition de NaCl (65 g/l). On répète trois fois cette opération, puis le produit final est dialysé contre l'eau et lyophilisé.

Le Tableau II indique les rendements en complexe actif obtenus à partir de poudre acétonique de post-hypophyses de quatre espèces. Ce rendement est exceptionnellement bon dans le cas de la poudre post-hypophysaire du mouton (92 % des activités initiales).

On constate que si les poudres acétoniques ont des teneurs en activité assez voisines (*cf.* Tableau I), les rendements en complexe actif varient d'un tissu à l'autre. Ceci est probablement dû aux différences de solubilité des neurophysines car c'est cette solubilité qui conditionne la précipitation du complexe. D'autre part il est à noter que les activités spécifiques des différents complexes sont du même ordre.

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* Les glandes ont été fournies par les Etablissements Choay que nous sommes heureux de remercier ici.

TABLEAU II

RENDEMENT EN COMPLEXE ACTIF*

Espèce	*Complexe actif* *g*	*Activité ocytocique* *unités/mg*	*Activité vasopressique* *unités/mg*
Boeuf	1.5	18–20	18–20
Porc	1.5	18	18
Cheval	0.8	20	10
Mouton	4.2	18	18

* Pour 100 g de poudre acétonique.

Dissociation du complexe actif

Le complexe est dissous dans l'acide acétique 0.25 % à une concentration de 1 %. On ajoute alors un volume convenable d'acide trichloracétique à 100 % (p/v) de façon à porter la concentration finale de la solution à 5 % en acide. La neurophysine est précipitée, mais les peptides demeurent dans le liquide surnageant. On sépare le précipité par centrifugation, et le culot est remis en suspension dans un volume égal d'acide acétique 0.25 %. On répète la précipitation dans les mêmes conditions et on sépare le précipité par centrifugation.

Neurophysine: Le précipité est remis en suspension dans l'eau, et dialysé plusieurs jours contre l'eau pour éliminer l'acide trichloracétique (pH final 4.7). Puis la suspension est lyophilisée. Le rendement est de 75 mg de neurophysine pour 100 mg de complexe.

La neurophysine est chromatographiée sur carboxymethylcellulose (CM-cellulose). On utilise une colonne de 1 × 15 cm initialement équilibrée avec un tampon acétate de sodium 0.1 *M* pH 4. 20 mg de neurophysine, dissous dans 20 ml de tampon acétate sont alors introduits dans la colonne. On établit ensuite un gradient de pH à l'aide d'un tampon acétate de sodium 0.1 *M* pH 5.2 (chambre de mélange: 50 ml). On collecte des fractions de 1 ml (débit 60 ml/h) et on détermine la présence des protéines par la réaction de FOLIN selon LOWRY *et al.*[11]. Le résultat de la chromatographie est indiqué par la Fig. 1.

Le constituant principal représente 70 % du matériel chromatographié. Les fractions de 140 à 170 ml sont réunies et la solution est dialysée contre l'eau. Le pH (5.2) est alors ajusté à 4 avec de l'acide acétique et on effectue à nouveau une chromatographie sur carboxyméthylcellulose dans les mêmes conditions. Le résultat est indiqué par la Fig. 2.

Ocytocine et vasopressine: Après précipitation de la neurophysine par l'acide trichloracétique, les deux solutions surnageantes qui contiennent les hormones (80 % des activités initiales) sont réunies et l'acide est éliminé par passage sur une colonne d'Amberlite IR-45 forme acétate (1.5 × 10 cm). On lave la colonne à l'eau, et les deux hormones sont quantitativement récupérées dans le filtrat (pH 3.5). La purification de l'ocytocine et de la vasopressine du mouton est réalisée par chromato-

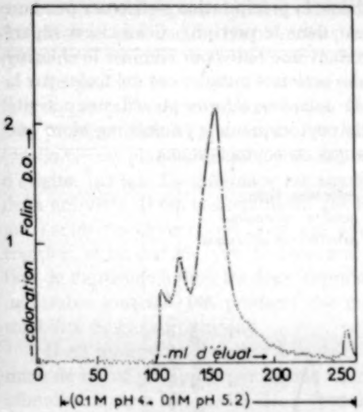

Fig. 1. 1ère chromatographie de la neurophysine sur carboxyméthylcellulose (colonne de 1 × 15 cm initialement équilibrée avec un tampon acétate de soude 0.1 *M* à pH 4. Gradient avec un tampon acétate 0.1 *M* pH 5.2. Chambre de mélange 50 ml).

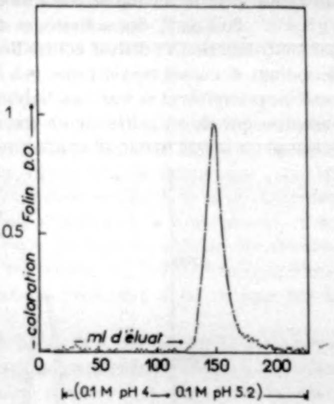

Fig. 2. 2ème chromatographie de la neurophysine sur carboxyméthylcellulose (conditions identiques à celles de la Fig. 1 ; détails dans le texte).

graphie sur colonne d'Amberlite IRC-50 dans des conditions déjà décrites[4]. La solution est passée sur l'échangeur forme H et les hormones demeurent adsorbées au sommet de la colonne. Celle-ci est équilibrée avec un tampon acétate d'ammonium 0.1 *M* à pH 5.0. L'ocytocine est d'abord éluée en établissant un gradient de pH et de force ionique avec une solution d'acétate d'ammonium 0.5 *M* pH 7.7 (chambre de mélange 50 ml) puis la vasopressine est éluée en élevant le gradient avec une solution d'acétate d'ammonium 0.75 *M* pH 7.7 (*cf.* Fig. 3).

Les tubes contenant respectivement les activités ocytocique et vasopressique sont rassemblés, et l'acétate d'ammonium est éliminé par évaporation sous vide. Les peptides ainsi obtenus paraissent chromatographiquement et électrophorétiquement purs[4].

Les comportements électrophorétiques des ocytocines du mouton et du cheval ainsi que ceux des vasopressines du mouton et du boeuf sont identiques comme l'indique le Tableau III.

L'étude des structures chimiques a montré que l'ocytocine et la vasopressine du mouton étaient respectivement identiques à l'ocytocine et à la vasopressine du boeuf et du cheval[4].

Reconstitution du complexe actif

On utilise une solution (2.4 ml) contenant 25 unités de vasopressine et 20 unités d'ocytocine/ml. Le pH est amené à 3.9 avec de la soude 0.1 *N*, et le volume est ajusté à 3 ml avec un tampon acétate de sodium *M* à pH 3.9. On dissout ensuite la neurophysine de précipitation à raison de 1 mg/ml: les proportions des activités vasopressique et ocytocique sont alors respectivement de 20 unités et 17 unités/mg de protéine.

On ajoute ensuite 100 mg de NaCl/ml et on laisse la précipitation s'effectuer pendant 11 h à 0°. 80 à 90 % des activités se trouvent dans le précipité. Celui-ci est séparé par centrifugation et dialysé contre l'eau pendant une nuit pour éliminer le chlorure de sodium. On constate alors que 75 à 80 % des activités initiales ont été fixées par la protéine précipitée et ne sont pas dialysables. Le complexe reformé possède une activité vasopressique de 15 unités/mg et une activité ocytocique de 13 unités/mg alors que le complexe initial titrait 18 unités vasopressique ou ocytocique/mg.

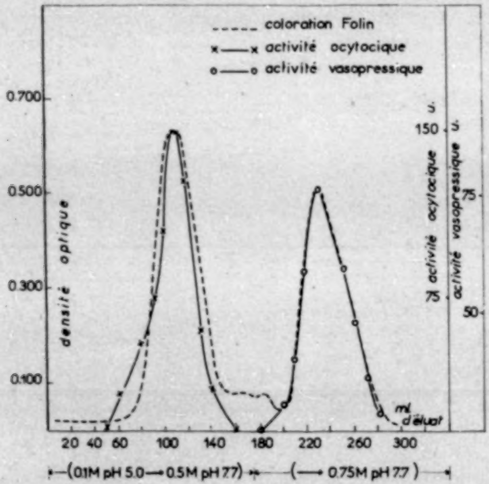

Fig. 3. Chromatographie de l'ocytocine et de la vasopressine sur Amberlite IRC-50. (Colonne de 0.9 × 10 cm équilibrée avec un tampon acétate d'ammonium 0.1 *M* pH 5.0. Gradient avec une solution d'acétate d'ammonium 0.5 *M* pH 7.7, puis 0.75 *M* pH 7.7. Chambre de mélange: 50 ml).

TABLEAU III

ELECTROPHORÈSE DE L'OCYTOCINE ET DE LA VASOPRESSINE DU MOUTON

Electrophorèse sur bandes de papier Whatman No. 1 (10 × 50 cm) sous une tension de 10 V/cm. Durée 5 h (pH 4: durée 4 h). Tampons: pH 4 (citrate–phosphate 0.05 *M*); pH 5 (acétate d'ammonium 0.05 *M*); pH 6.5 (acétate de pyridine 0.06 *M*); pH 8.6 (véronal–soude 0.06 *M*). Cheminements en mm vers la cathode, mesurés par rapport à la dinitrophényl éthanolamine.

	pH 4	*pH 5*	*pH 6.5*	*pH 8.6*
Ocytocine du mouton	71	81	26	2
Ocytocine du cheval	71	78	25	3
Vasopressine du mouton	103	123	71	65
Vasopressine du boeuf	101	123	71	60

DISCUSSION

Deux arguments plaident en faveur de la spécificité de l'adsorption de l'ocytocine et de la vasopressine sur la neurophysine: (a) L'absence de peptides étrangers adsorbés sur cette protéine lorsque le complexe a été purifié par plusieurs reprécipitations au moyen de chlorure de sodium. En effet la vasopressine et l'ocytocine apparaissent comme les seuls peptides présents dans le liquide surnageant lorsque le complexe a été dissocié par l'acide trichloracétique, et la neurophysine éliminée (*cf.* Fig. 3). (b) La reconstitution de l'association à partir des deux hormones et de la neurophysine.

En général, le complexe reformé possède des activités spécifiques un peu plus faibles que celles du complexe initial. Les peptides semblent moins fortement liées à la protéine dans le premier cas: le rendement en activités du complexe reformé (75–80 %) est plus faible que celui constaté lors de la préparation du complexe actif d'origine (92 %). La différence est significative, et va dans le même sens pour les deux activités. Il est vraisemblable que la neurophysine résultant de la précipitation par l'acide trichloracétique n'est pas absolument identique à la neurophysine d'extraction, et un certain type de dénaturation a pu se produire entraînant une diminution de l'aptitude à fixer les deux peptides. Le traitement par l'acide trichloracétique ne semble toutefois pas produire des modifications profondes si on en juge par la solubilité de la neurophysine.

Il est intéressant de noter à ce sujet que SCHWERT[12] a montré que la sérumalbumine de boeuf précipitée par l'acide trichloracétique paraissait identique à la sérumalbumine initiale lorsque les deux produits étaient comparés par ultracentrifugation, électrophorèse, par les courbes de solubilité et l'aptitude à cristalliser.

L'examen du Tableau II indique que les différents complexes obtenus à partir des post-hypophyses de différentes espèces possèdent des activités ocytocique et vasopressique égales, et constantes d'un produit à l'autre à l'exception du complexe obtenu à partir de glandes de cheval. Au lieu de posséder les deux activités avec une teneur de l'ordre de 18 unités/mg, ce produit a une activité ocytocique de 20 unités/mg et une activité vasopressique de 10 unités/mg. Chez le boeuf, le cheval et le mouton, on trouve la même ocytocine et la même vasopressine. La différence doit donc porter sur la neurophysine qui chez le cheval paraît particulière. Pour élucider cette question, nous avons effectué la reconstitution du complexe, en utilisant la neurophysine du cheval. L'expérience a été conduite dans les conditions décrites ci-dessus, mais en utilisant 1 mg de protéine de cheval par ml au lieu de 1 mg de protéine de boeuf. Le complexe reformé possède une activité ocytocique de 18 unités/mg et une activité vasopressique de 11 unités/mg soit pratiquement les activités spécifiques du complexe d'origine, avec le même rapport. D'autre part, au cours de la chromatographie sur CM-cellulose, la neurophysine du cheval a un comportement différent des neurophysines du boeuf et du mouton qui paraissent semblables à ce point de vue. L'étude des différentes neurophysines est actuellement en cours.

RÉSUMÉ

A partir de post-hypophyses de mouton a été obtenu un complexe actif renfermant 90 % des activités ocytocique et vasopressique de la glande et formé par l'association de l'ocytocine et de la vasopressine avec une protéine, la neurophysine. Le complexe a été dissocié par l'acide trichloracétique et les trois substances ont été purifiées par chromatographie. La reconstitution du complexe à partir de ses éléments a été assurée en respectant les conditions de formation. Des complexes analogues ont été obtenus à partir de glandes de boeuf, de cheval et de porc; il est possible de réaliser des associations interspécifiques entre hormones et neurophysines d'origines différentes.

REMERCIEMENT

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A COMPARISON OF SOME *VIC* GLYCOL DEHYDROGENASE SYSTEMS FOUND IN *AEROBACTER AEROGENES**

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SUMMARY

Two DPN-requiring *vic* glycol dehydrogenases can be isolated from extracts of a variant of *A. aerogenes*. Deamino DPN can replace the DPN requirement of enzyme A (obtained from cells grown on a glycerol-salts medium) and the 3-acetylpyridine analogue of DPN can replace the DPN requirement of enzyme B (obtained from cells grown on a glucose-salts media). Deamino DPN cannot replace the DPN requirement of enzyme B nor can the 3-acetylpyridine analogue substitute for DPN with enzyme A.

Enzymes A and B can be distinguished by properties other than pyridine nucleotide specificity. For example, they can be identified by differences in stability and in pH optima. Finally, enzyme A was found to be inducible.

Though enzymes A and B are physically inseparable, they are immunologically unrelated since no cross reactions take place.

Several closely related strains of bacteria were found to differ widely in their ability to carry out DPN (and DPN analogue) mediated oxidations of *vic* glycols.

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INTRODUCTION

The properties of a partially purified glycerol dehydrogenase obtained from *Aerobacter aerogenes* (American Type Culture Collection 8724) have been described by BURTON AND KAPLAN[1,2]. This DPN* specific enzyme catalyzes the oxidation of glycerol to dihydroxyacetone. Other *vic* glycols, for example, 1,2-propanediol and 2,3-butanediol are oxidized to the corresponding ketone. STRECKER AND HARARY[3] have studied a butanediol dehydrogenase from *Escherichia coli*, and pyridine nucleotide linked glycol dehydrogenases from *Acetobacter suboxydans* have been described by GOLDSCHMIDT AND KRAMPITZ[4] and VIRTANEN AND NORDLUND[5].

A variant of *A. aerogenes* (ATCC No. 8724) has been isolated which contains an enzyme having a general *vic* glycol specificity and a requirement for DPN. Of particular interest is the observation that *A. aerogenes* (var.) grown on glycerol as the sole carbon source forms a *vic* glycol dehydrogenase capable of substituting deamino DPN for DPN. When the organism is grown in a medium containing glucose as the sole carbon source, deamino DPN is no longer capable of substituting for DPN when assayed with the *vic* glycol dehydrogenase. This paper describes these glycol dehydrogenases and compares some of the properties of these enzymes.

MATERIALS AND METHODS

Glycerol was purchased from Merck and Company, dihydroxyacetone from Nutritional Biochemicals Corporation, and 1,2-propanediol (propylene glycol) and 2,3-butanediol (2,3-butylene glycol) from Fischer Chemical Company. Acetol (hydroxyacetone) was synthesized according to the method of LEVENE AND WALTI[6]. Lactaldehyde was synthesized according to the method of DWORZAK AND PRODINGER[7]. The 3-acetylpyridine and the 3-pyridine aldehyde analogues of DPN were prepared by the exchange reaction with the pig brain DPNase as described by KAPLAN AND STOLZENBACH[8] and deamino DPN by the action of nitrous acid on DPN[9]. *A. aerogenes* (ATCC No. 8724) was grown on a medium according to DAGLEY *et al.*[10] consisting of 0.54 % KH_2PO_4, 0.12 % $(NH_4)_2SO_4$, 0.04 % $MgSO_4 \cdot 7H_2O$ made up to volume with distilled water. The carbon source was 1.5 % glucose or glycerol (as indicated), and the final pH 7.1 (30°). A 25-ml actively growing inoculum (of approx. $1 \cdot 10^8$ cells/ml) was used per 20 l when the bacteria were grown on glucose, and a 50-ml inoculum per 20 l was used when the bacteria were grown on glycerol.

Enzyme unit of activity

One unit of enzyme is defined as that amount of protein which causes an initial rate of change in O.D. (at 340 mμ) of 1.0 per min under the conditions employed. The specific activity is expressed as units/mg of protein.

Enzyme assay

0.5 mmole of 1,2-propanediol or glycerol and 1.2 μmoles of DPN are diluted with 2.7 ml of sodium pyrophosphate solution (0.1 *M*, pH 9.2). After initially deter-

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[*] The following abbreviations are used: DPN, diphosphopyridine nucleotide; DPNH, the reduced form of diphosphopyridine nucleotide; deamino DPN, the deaminated analogue of diphosphopyridine nucleotide.

mining the O.D. at 340 mμ, the reaction is started by the addition of approximately 0.2 unit of enzyme.

Acetol was measured fluorometrically according to the method of MILLER AND HUGGINS[11].

Because of the multiplicity of enzyme activities measured we shall refer to glycol dehydrogenase enzyme systems A and B (or simply enzymes A and B). These enzymes will be operationally defined in terms of their enzymic activities and source as shown in Table I.

TABLE I

DEFINITION OF ENZYME SYSTEMS USED IN THE TEXT

	Growth medium	*Substrate and pyridine nucleotide requirements for enzymic activity*
Enzyme A	glycerol plus salts	1,2-propanediol plus deamino DPN 2,3-butanediol plus deamino DPN Glycerol plus DPN 1,2-propanediol plus DPN 2,3-butanediol plus DPN
Enzyme B	glucose plus salts	Glycerol plus DPN 1,2-propanediol plus DPN 2,3-butanediol plus DPN

EXPERIMENTAL

Purification procedure for enzyme A

Step 1: 50 g of wet cells grown in the glycerol-salts media (obtained from 20 l) were suspended in 150 ml of cold distilled water and disrupted in a Raytheon sonic oscillator (10 kc) for 30 min. The cell debris was removed by centrifugation in a refrigerated Servall centrifuge (3°) for 15 min at maximum speed. The supernatant solution was poured off and diluted to 490 ml. Since the enzymic activity of the extract was found to be extremely labile (complete loss of activity in 24 h), it was necessary to proceed with step 2 immediately.

Step 2: Solid $(NH_4)_2SO_4$ was slowly added to the stirred protein solution (5°, pH maintained at 7.0–7.2 with concentrated NH_4OH) to give a final saturation of 50% with respect to $(NH_4)_2SO_4$. After a 15-min equilibration period, the small amount of precipitate was removed by centrifugation (3°) and discarded. Ammonium sulfate was added to the supernatant solution to a final concentration of 85%. After equilibration and centrifugation, this large precipitate was extracted 3 times with cold TRIS buffer (tris(hydroxymethyl)aminomethane 0.03 *M*, pH 8.0), and the insoluble material was removed by high speed centrifugation. The clear, yellowish supernatant solution contained the enzymic activity (total volume 35 ml). The preparation was stored at —20°.

It has been found that crude extracts contain a very active DPNH oxidase. Since the enzymic activity is defined in terms of the rate of formation of DPNH, the enzymic activity observed in step 1 is low because the DPNH formed in the dehydrogenase reaction is reoxidized by the contaminating oxidase. Separation of this DPNH oxidase activity by ammonium sulfate treatment caused the apparent increase in total units of activity recoverable as shown in step 2 of Table II.

TABLE II

PURIFICATION OF ENZYME SYSTEM A*

	Protein (mg)	*Units/ml*	*Total units*	*Specific activity (units/mg protein)*	*Purification*	*% recovery*	*a/c****	*b/c****
1. Crude extract								
a. 1,2-propanediol plus DPN**	7.14	0.68	338	0.10				
b. 1,2-propanediol plus deamino DPN**	7.14	0.22	109	0.03	—	—	2.0	0.6
c. glycerol plus DPN**	7.14	0.38	189	0.05				
2. $(NH_4)_2SO_4$ precipitate								
a. 1,2-propanediol plus DPN	15.5	23.0	805	1.48	14.8	238§		
b. 1,2-propanediol plus deamino DPN	15.5	10.0	350	0.65	21.7	321§	1.4	0.6
c. glycerol plus DPN	15.5	16.5	578	1.07	21.7	305§		
3. $MnCl_2$ supernatant								
a. 1,2-propanediol plus DPN	5.5	6.8	585	1.24	12.4	173		
b. 1,2-propanediol plus deamino DPN	5.5	2.6	224	0.47	15.3	205	1.3	0.5
c. glycerol plus DPN	5.5	5.3	456	0.96	19.2	241		
4. Combined $Ca_3(PO_4)_2$ washings								
a. 1,2-propanediol plus DPN	1.04	3.7	259	3.56	35.6	77		
b. 1,2-propanediol plus deamino DPN	1.04	1.6	114	1.54	51.3	104	1.1	0.5
c. glycerol plus DPN	1.04	3.4	238	3.27	65.4	126		
5. Supernatant after heating								
a. 1,2-propanediol plus DPN	0.47	2.8	185	5.96	59.6	55		
b. 1,2-propanediol plus deamino DPN	0.47	0.7	46	1.49	49.6	42	1.3	0.3
c. glycerol plus DPN	0.47	2.1	139	4.47	89.4	74		

* Details are described in the text.

** a, b and c (1,2-propanediol plus DPN, etc.) are the substrate-coenzyme combinations used in assaying enzymic activity.

*** a/c and b/c are ratios of the specific activities of 1,2-propanediol plus DPN to glycerol plus DPN, and of 1,2-propanediol plus deamino DPN to glycerol plus DPN.

§ The apparent recovery of greater than 100% enzymic activity is due to removal of a DPNH oxidase activity present in the crude extract (see text for details).

Step 3: Nucleic acid can be removed from extracts of *A. aerogenes* by employing manganese chloride according to the method of KORKES *et al.*[12]. The protein solution was diluted to a concentration of 6 mg/ml with cold TRIS buffer (0.2 *M*, pH 7.9 total volume 86 ml), placed in an ice bath and stirred continuously while 3.5 ml of manganese chloride (1.0 *M*) was added dropwise. After a 10-min equilibration period, the precipitate was removed by centrifugation. The supernatant (86 ml, 5.5 mg protein/ml) was found to contain most of the enzymic activity.

Step 4: Calcium phosphate gel was added to the protein solution to give a final gel-protein ratio of 2:1. After equilibration, the gel was removed by centrifugation and washed 3 times with cold potassium phosphate buffer (0.05 *M*, pH 7.5, 70 ml total volume of the combined washes). The washings were found to contain most of the activity.

Step 5: 10-ml portions of the enzymically active solution were placed in a 50-ml Erlenmeyer flask and heated in a 70° water bath for 2 min then rapidly cooled in an ice bath. The denatured protein was removed by centrifugation and the supernatant solution (60 ml) was lyophilized. The powder was dissolved in cold TRIS buffer (0.05 *M*, pH 7.5, 6.6 ml) and centrifuged at high speed to remove insoluble material. The protein solution was divided into two parts for subsequent dialysis and storage. 1,2-propanediol was added to one portion to a final concentration of 20 % (v/v) while the remaining solution was diluted with glycerol to a final concentration of 20 % (v/v). The former solution was dialyzed for 24 h against TRIS-20 % 1,2-propanediol (pH 7.5), while the latter was dialyzed against TRIS-20 % glycerol (pH 7.5) to remove salt, and other dialysable impurities. The solutions were stored frozen for several months without loss of enzymic activity.

It was suspected that the more general diol dehydrogenase enzymic specificity of glycerol grown bacteria might be due to the combined effect of several enzyme systems. The purification of enzymes derived from glycerol grown bacteria offers no support for this hypothesis. It can be seen in the last column of Table II that during the course of purification, there is no appreciable change in the ratio of specific activities. However, one cannot conclude from the results of the purification that there is only one enzyme present, for all proteins with similar physical properties can be expected to behave similarly on purification. Experiments dealing with the number of enzymes in this system, their origin and synthesis, will be described later.

Purification procedure for enzyme B

This enzyme was partially purified according to the method of BURTON[2], and a summary of the purification achieved is shown in Table III. As previously stated, the enzyme differs from enzyme A in that it is incapable of substituting deamino DPN for DPN (see Table VI for coenzyme specificity). The glycerol dehydrogenase which is present in the parent strain of *A. aerogenes* No. 8724 has not been identified in the variant isolated and studied herein. It should be noted that enzyme B was partially purified by the same procedure described by BURTON for the purification of the glycerol dehydrogenase identified and studied in the parent strain. Table III lists the specific activities calculated for both glycerol and 1,2-propanediol. The ratio of these specific activities suggests that there may be some slight contamination of the enzyme B by the glycerol dehydrogenase (originally purified by BURTON) or of some other *vic* glycol dehydrogenases. In addition to the enzymes described, there

TABLE III

PURIFICATION OF ENZYME SYSTEM B*

Fraction	*Specific activity (units/mg)***		*a/b****
	a *Glycerol*	*b* *1,2-propanediol*	
1. Cell-free extract	0.4	0.4	1.0
2. 0–40% saturated ammonium sulfate supernatant	2.1	3.2	0.65
3. Eluate of 0–40% saturated ammonium sulfate precipitate	16.1	19.2	0.84
4. Calcium phosphate gel (20 mg/ml) eluate from step 3	14.2	23.6	0.60
5. Heating at 60° for 5 min	22.5	29.5	0.75
Overall purification	57 ×	74 ×	—

* Obtained from cells grown on a glucose–salts medium.

** Purification and assay were conducted according to the method of BURTON[2]. One unit of enzyme activity is defined as that amount of enzyme which causes an initial rate of change in O.D. at 340 mμ of 1.0/min under the conditions of the assay. The specific activity is expressed as units of activity/mg of protein. Assay conditions are described in MATERIALS AND METHODS.

*** a/b is the ratio of the specific activities of glycerol plus DPN to that of 1,2-propanediol plus DPN.

TABLE IV

STABILITY OF ENZYME SYSTEMS A AND B

Enzyme A was obtained from bacteria grown in a glycerol-salts media and enzyme B was obtained from bacteria grown in a glucose-salts media.

	*Activity**			
	Enzyme A		*Enzyme B*	
	Units	*% activity*	*Units*	*% activity*
Crude extract control	0.685	100	0.590	100
Storage (24 h, —15°)**	0.034	5	0.431	73
Storage (14 days, —15°)	0	0	0.283	48
Storage (3 months, —15°), in 0.1 *M* TRIS, 20% 1,2-propanediol	0.630	92	0.607	103
Dialysis (24 h, 3°)***	0.010	1	0.212	36

* The assay is the same as defined previously under "enzyme assay". The activities are recorded in terms of change in O.D. (at 340 mμ) per min. The activities are recorded for 1,2-propanediol plus DPN. Other substrate-pyridine nucleotide combinations normally assayed with enzyme A give the same results.

** The enzyme was diluted to a concentration of 10 mg/ml with sodium pyrophosphate (0.1 *M*, pH 9.3), sodium phosphate (0.05 *M*, pH 7.5) and TRIS (0.1 *M*, pH 7.0).

*** Dialysis was carried out against distilled water, potassium phosphate (0.05 *M*, pH 7.5) and Versene ($1 \cdot 10^{-3}$ *M*).

is present in extracts of *A. aerogenes* No. 8724 a *vic* glycol dehydrogenase specific for 2,3-butanediol and DPN. The 2,3-butanediol dehydrogenase can be separated from the other *vic* glycol dehydrogenases by ammonium sulfate fractionation[13].

Enzyme stability

As seen in Table IV crude extracts containing enzyme A prepared from glycerol grown cells were found to have an enzymic half-life of approximately 1 day or less regardless of the temperature of storage (one preparation was found to be completely inactive after 12 h storage at —15°). In contrast, extracts of enzyme B prepared from glucose grown cells retained 50 % of their enzymic activity after two weeks storage at —15°. The partially purified enzyme A which is stabilized with 1,2-propanediol or glycerol was found to retain complete activity after 3 months storage at —15° despite the fact that the preparation had been repeatedly frozen and thawed. Enzyme A, when not protected by the addition of glycols, completely lost enzyme activity after a 24-h dialysis against distilled water or dilute phosphate buffer (0.1 *M*, pH 7.5). Enzyme B retained 36 % of its original activity under the same conditions.

Product of 1,2-propanediol oxidation

BURTON found that *A. aerogenes* oxidized glycerol according to eqn. (1).

$$\mathrm{CH_2OHCHOHCH_2OH + DPN^+ \rightleftharpoons HOCH_2COCH_2OH + DPNH + H^+} \qquad (1)$$

It has been stated that chemically synthesized acetol (hydroxyacetone) but not lactaldehyde could participate in the reverse reaction. Utilizing the method of HUGGINS AND MILLER[11], it is possible to show that the product of the oxidation of 1,2-propanediol is acetol and that acetol and DPNH are produced in equimolar amounts. The reaction can be formulated according to eqn. (2).

$$\mathrm{CH_3CHOHCH_2OH + DPN^+ \rightleftharpoons CH_3COCH_2OH + DPNH + H^+} \qquad (2)$$

The results of this analysis are presented in Table V. The relationship between DPN reduction and acetol formation was demonstrated by measuring DPNH spectrophotometrically, then the reaction was stopped with trichloroacetic acid (10 % final concentration), and the acetol was measured fluorometrically. From the controls one can see that substrate, pyridine nucleotide and enzyme are all required for the reaction.

TABLE V

OXIDATION OF 1,2-PROPANEDIOL

Constituents	*Acetol formed** (*μmoles*)	*DPNH formed*** (*μmoles*)
Complete***	0.23	0.21
Minus 1,2-propanediol	0.035	0
Minus enzyme	0	0
Minus DPN	0.015	0

* Measured according to the method of HUGGINS AND MILLER[11].

** Determined spectrophotometrically at 340 mμ assuming a molar absorbancy index (a_M) of[16] $6.22 \cdot 10^3$. a_M is the molar absorbancy index (nomenclature recommended by the Bureau of Standards, circular LC 857, May 19, 1947) and is defined as the absorbancy ($-\log_{10}$ transmission) divided by the concentration (moles/l).

*** Complete system described in enzyme assay.

Characteristics of the partially purified enzymes A and B

Activity of analogues of pyridine nucleotides: Several pyridine nucleotide analogues of DPN are capable of substituting for DPN in the partially purified *vic* glycol dehydrogenase systems (see Table VI). The pyridine-3-aldehyde analogue of DPN is active with both enzymes A and B and seems to have some specificity for 1,2-propanediol as substrate. The 3-acetylpyridine analogue of DPN is active only with enzyme B

TABLE VI

ENZYMIC ACTIVITY OF PYRIDINE NUCLEOTIDE ANALOGUES

500 μmoles of glycerol or 1,2-propanediol and pyridine nucleotide as indicated were diluted to a final volume of 3.0 ml with sodium pyrophosphate buffer (0.1 *M*, pH 9.3). The reaction was started with 1 unit of the appropriate enzyme, and the rate of reduction of the pyridine nucleotide was measured every 15 sec at the following wavelengths: DPN, 340 mμ; deamino DPN, 340 mμ; 3-acetylpyridine analogue of DPN, 365 mμ; deamino-3-acetylpyridine DPN, 365 mμ; pyridine-3-aldehyde, 355 mμ; deamino-pyridine-3-aldehyde DPN, 355 mμ; nicotinic acid DPN, 330 mμ.

	*Activity (%)**			
	Enzyme B		*Enzyme A*	
	1,2-propanediol	*Glycerol*	*1,2-propanediol*	*Glycerol*
DPN (1 μmole/ml)	100*	15	100*	67
Deamino DPN (2 μmoles/ml)	5(12)**	3(10)**	16(252)**	4(98)**
3-acetylpyridine analogue of DPN (1 μmole/ml)	73	3	4	1
Deamino-3-acetylpyridine analogue of DPN (2 μmoles/ml)	1	1	0	0
Pyridine-3-aldehyde DPN (1 μmole/ml)	55	6	20	13
Deamino-pyridine-3-aldehyde (2 μmoles/ml)	3	0	0	0
Nicotinic acid analogue (2 μmoles/ml)	0	0	0	0

* The enzymic activity of the various analogues is reported in % activity relative to the activity measured with DPN and 1,2-propanediol.

** The values given in the parentheses were obtained when the concentration of deamino DPN was increased to 9 μmoles/ml.

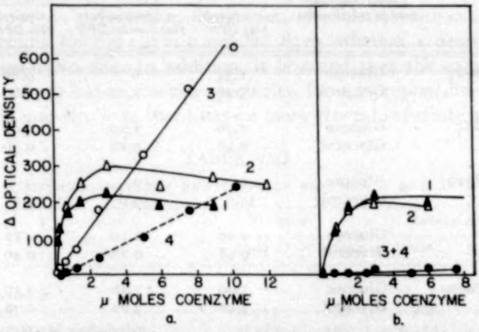

Fig. 1. Rate studies of glycerol and glucose grown *A. aerogenes* extracts. (a): glycerol grown cells; (b): glucose grown cells. 100 μmoles of glycerol or 1,2-propandediol, 0.1 *M* potassium pyrophosphate (pH 9.3) and the glucose or glycerol grown crude bacterial extract (approximately 0.5 units) was added and the reaction started by the addition of coenzyme (final volume, 3.0 ml; 25°). The change in O.D. at 340 mμ in the first 2 min was observed as a function of coenzyme concentration. The substrate and pyridine nucleotide combinations used are as follows: curve 1, glycerol plus DPN; curve 2, 1,2-propanediol plus DPN; curve 3, 1,2-propanediol plus deamino DPN; curve 4, glycerol plus deamino DPN.

and will not substitute for DPN in the presence of enzyme A. Deamino DPN is active only with enzyme A and not enzyme B. Though the deamino DPN activity is only 16% of the DPN activity in this particular experiment, high concentrations of deamino DPN have considerably higher activity than the natural coenzyme with enzyme A, but very little activity with enzyme B (Table VI). The acetyl pyridine

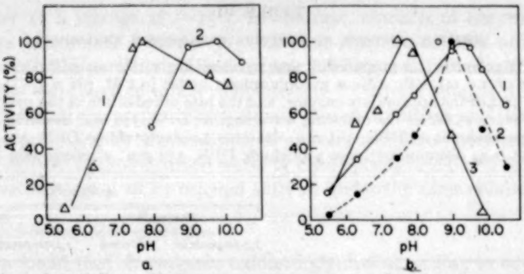

Fig. 2. pH optima of partially purified enzyme systems. 500 μmoles of 1,2-propanediol or glycerol and 0.63 μmole of DPN or deamino DPN were diluted with sodium phosphate (0.2 *M*, 25°, final volume, 3.0 ml) which was adjusted to the pH value shown on the abscissa. The reaction was started with 100 μg of enzyme protein and the reduction of pyridine nucleotide (at 340 mμ) was measured for the first 2 min of reaction. Maximal activity for each substrate–coenzyme combination is arbitrarily set at 100, other activities of this combination being expressed as % of the maximum. pH optima are plotted for 1,2-propanediol plus DPN (curve 1), glycerol plus DPN (curve 2), and 1,2-propanediol plus deamino DPN (curve 3). (a): enzymes from glucose grown cells. (b): enzymes from glycerol grown cells.

TABLE VII

A SURVEY OF SOME GLYCOL DEHYDROGENASES

Bacteria	*Carbon compound used in growth media*	*Enzymic activity (units/ml)**			
		1,2-propanediol plus DPN	*1,2-propanediol plus deamino DPN*	*Glycerol plus DPN*	*Glycerol plus deamino DPN*
A. aerogenes No. 8724	Glucose	1.34	0	0.86	0
	Glycerol	5.46	1.00	4.60	0.08
A. aerogenes No. 884	Glucose	0.76	1.20	0.80	0.90
	Glycerol	0.28	0.18	0.36	0.12
A. aerogenes No. 8329	Glucose	0	0	0	0
	Glycerol	35.0	39.0	12.2	4.30
E. coli K-12	Glucose	0.20	0.10	0.12	0.08
	Glycerol	0.48	0.28	0.40	0.24
Acetobacter suboxydans	Glucose	1.00	1.58	0.12	0.22
	Glycerol	2.48	3.82	0.78	0.64

* 500 μmoles of 1,2-propanediol or glycerol and DPN (1.2 μmoles) or deamino DPN (6.1 μmoles) were diluted with sodium pyrophosphate (0.1 *M*, pH 9.3, 25°) to give a final volume of 3.0 ml. The reaction was started with approximately 1 mg of enzyme protein, the rate of reaction being followed by the reduction of pyridine nucleotide at 340 mμ. The various combinations of substrate and pyridine nucleotide assayed are listed at the top of each column. Activity measurements are recorded in terms of units/ml of crude sonic extract which contained approximately 10 mg of protein/ml estimated by the method of WARBURG AND CHRISTIAN.

analogues of DPN and deamino DPN have a substrate specificity largely for 1,2-propanediol.

Fig. 1 shows that the K_m for deamino DPN with enzyme A is infinity in the concentration range from 0.1 to 10 μmoles/ml (using 1,2-propanediol as substrate) and that within this concentration range, the rate of reduction of deamino DPN increases with increasing concentration of this analogue. At high concentrations (10 μmoles/ml), the initial rate of reaction is 3 to 5 times greater than the rate observed with DPN. It would therefore seem that deamino DPN and the 3-acetylpyridine analogue of DPN can be used as "chemical tracers" in identifying enzymes A and B since deamino DPN can substitute for DPN only with enzyme A while the 3-acetylpyridine analogue of DPN can substitute for DPN only with enzyme B.

pH optimum: The diol dehydrogenases (enzymes A and B) differ not only in coenzyme specificity but also in the pH optimal enzyme activity as can be seen in Figs. 2a and 2b. For example, enzyme B, assayed with glycerol and DPN (Fig. 2a) exhibits a broad pH optimum (from 9–10) compared with enzyme A which has a sharp maximum at pH 9. This difference in the pH optimum (for the two systems studied) reflects changes in the physical structure of the two diol dehydrogenases. Preliminary information, to be presented later in this paper, indicates that these diol dehydrogenases are immunochemically different.

Strains of bacteria: Table VII summarizes the results of a study made to determine the ubiquity of these enzyme systems in some related strains of bacteria. When the strains of *A. aerogenes* are grown on a glucose medium, marked differences in enzymic activity are noted. Strain No. 8724 (var.) (the strain which we have studied in detail) cannot substitute deamino DPN for DPN and extracts of this bacterium are most active when 1,2-propanediol is the substrate. Strain No. 884 has a general specificity with regard to both substrate and pyridine nucleotide and strain No. 8329 has no *vic* glycol dehydrogenase activity at all when grown on glucose. When these same organisms are grown on a glycerol medium a considerable increase in enzyme activity is noted with strains No. 8724 (var.) and No. 8329, whereas a decrease in activity is noted with strain No. 884. In addition, it is noted that the extract of glycerol grown strain No. 8329 has a marked specificity for 1,2-propanediol while extracts of strains No. 884 and No. 8724 (var.) have a more general substrate specificity when

TABLE VIII

ANTIGEN-ANTIBODY REACTIONS OF ENZYMES A AND B

	DPN		*Deamino DPN*	
	Units	*% activity*	*Units**	*% activity*
A. Enzyme A				
Alone	0.271	100	0.160	100
+ control γ-globulin	0.278	103	0.173	108
+ anti-enzyme A γ-globulin	0.107	39	0.043	27
+ anti-enzyme B γ-globulin	0.260	96	0.160	100
B. Enzyme B**				
Alone	0.142	100	—	—
+ control γ-globulin	0.142	103	—	—
+ anti-enzyme A γ-globulin	0.159	112	—	—
+ anti-enzyme B γ-globulin	0.153	108	—	—

* 0.2 mg of enzyme protein and 0.1 ml of the appropriate γ-globulin (except for the enzyme controls) were preincubated for 15 min at 37°. 500 μmoles of 1,2-propanediol and DPN (1.2 μmoles) or deamino DPN (6.1 μmoles) were diluted with sodium pyrophosphate (0.1 *M*, pH 9.3, 25°) to give a final volume of 3.0 ml. The reaction was started with the preincubated enzyme, γ-globulin mixture and the change in O.D. (at 340 mμ) was recorded for the first 2 min of reaction.

** Enzyme B does not catalyse the reaction between deamino DPN and 1,2-propanediol.

grown in a glycerol medium. The K-12 strain of *E. coli* has a general low level of *vic* glycol dehydrogenase activity and *Acetobacter suboxydans* shows a greater specificity for 1,2-propanediol as substrate regardless of the carbon source used in the growth medium. The results are of interest since there seems to be no common enzyme system in these organisms for the direct metabolism of *vic* glycols. The fact that several strains of the same organism differ widely in their capacity to metabolize *vic* glycols is noteworthy.

Immunochemical studies of enzymes A and B: Antisera to enzymes A and B were produced in rabbits by intravenous injection of alum-precipitated proteins. Nine injections, one every other day, were given. The doses were from 0.2 to 0.7 mg of protein. The rabbits were rested 1 month and a second course of injections were administered. The rabbits were exsanguinated 6 days after the last injection. Sera were cleared by centrifugation, heated for 30 min at 56° to inactivate complement and fractionated with ammonium sulfate to obtain the γ-globulin according to the method of STERNBERGER AND PETERMAN[14]. 0.1 ml of the γ-globulin fractions were preincubated with enzymes A and B for 15 min at 37°. The results are presented in Table VIII. Enzyme B does not appear to be antigenic, since no inhibition of enzyme activity is observed and no enzyme activity was precipitated on standing in the cold (3°) for 24 h. Anti-enzyme A selectively inhibits enzyme A but does not inhibit enzyme B activity. It is of interest to note that the antisera inhibit both the DPN and deamino DPN activities of enzyme A.

DISCUSSION

Crude extracts of *A. aerogenes* grown in a medium containing both glucose and glycerol exhibited *vic* glycol dehydrogenase activity which we believed to be due to multiple enzyme systems. Extracts of cells grown in a medium containing glycerol as the sole carbon source exhibited a general *vic* glycol activity. When grown in this carbon source deamino DPN was not only active in the system but at high concentrations exceeded the rate of reaction with DPN. In contrast, extracts of cells grown on glucose as the sole carbon source showed a specificity for DPN. We have not been able to find deamino DPN in extracts of glycerol grown bacteria.

Based on the enzymes elicted when bacteria were grown on glucose or glycerol, it was expected that cells initially grown in a glucose medium and subsequently transferred to a glycerol medium would contain both enzymes A and B. Kinetic studies (mixed substrate at saturation) have not revealed the presence of both enzymes. We have had no success in separating enzymes A and B found in extracts of *A. aerogenes* grown on the mixed carbon source medium, nor can the partially purified enzymes be separated when artificially mixed. However, a few differences have been noted in extracts (and partially purified enzymes) of bacteria grown on either glycerol or glucose. For example, the enzyme obtained from glycerol grown bacteria is much more unstable during storage and dialysis. Other differences noted relate to pyridine nucleotide specificity and pH optima for enzymic activity. When glucose grown bacteria are induced by the presence of glycerol (and in the absence of glucose), the enzyme activities produced are indistinguishable from the system obtained by growing the bacteria solely on glycerol. It is suspected that these two glycol dehydrogenases are different proteins having sufficiently similar properties to make physical separation difficult. The fact that there is no immunochemical cross reaction indicates that these two proteins are not structurally identical. The properties of the inducible *vic* glycol dehydrogenase are described in the next article.

PULLMAN *et al.*[15] have compared the activity of DPN with deamino DPN in a number of enzyme systems and concluded that differences in rates reflect a difference in affinities of some of the reactants. We have concluded that deamino DPN had a much lower affinity for glycol dehydrogenase A than DPN. At high concentrations of deamino DPN the rate which is observed is 3 times faster than with DPN when 1,2-propanediol is the substrate. When deamino DPNH or DPNH were assayed with acetol (hydroxyacetone), the reduced deamino DPN rate again exceeded that of DPNH. Lactaldehyde was inactive in this system.

ACKNOWLEDGEMENTS

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ADAPTIVE FORMATION OF A *VIC* GLYCOL DEHYDROGENASE IN *AEROBACTER AEROGENES*[*]

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SUMMARY

Glycerol is capable of inducing a *vic* glycol dehydrogenase in cells which were originally grown on a glucose or lactate medium. The rate of induced enzyme formation can be measured by using a pyridine nucleotide analogue of DPN, deamino DPN, since the constitutive DPN-linked 1,2-propanediol dehydrogenase is incapable of reacting with this analogue.

Detectable induced enzyme synthesis occurs after a lag of 15 min. The formation of induced enzyme is specifically dependent upon glycerol; other *vic* glycols and dihydroxyacetone are inert as inducers.

The experimentally determined concentration of glycerol required for half maximal enzyme induction is $0.8 \cdot 10^{-2}$ *M*. Other necessary requirements for induced enzyme formation are a nitrogen source, and an energy source.

Induced enzyme formation is inhibited by chloramphenicol, azaserine, potassium cyanide, sodium azide and dinitrophenol. The azaserine inhibition can be partially overcome by the aromatic amino acids but not by aliphatic amino acids, purines, pyrimidines, ribosides and ribotides.

INTRODUCTION

The problem of identifying and separating physically dissimilar proteins poses few problems today. Identification of physically similar proteins generally requires more elegant methodology. Thus, COHN AND TORRIANI[1,2] employed immunochemical methods for the identification and study of the "PZ" and "GZ" proteins originally found in *E. coli*, and SOBER *et al.*[3] have developed special chromatographic techniques for the isolation of several proteins from "homogeneous" serum protein fractions. We have been studying glycol dehydrogenase systems in *Aerobacter aerogenes* which have both physical and functional similarity[4].

When this organism is grown on a medium containing glucose and salts, an enzyme is produced which has glycol dehydrogenase activity with specificity for *vic* glycols and for DPN (enzyme B)[*], [**], [***]. If this same organism is grown on a medium containing glycerol plus salts, a *vic* glycol dehydrogenase system is produced which has a more general substrate specificity (enzyme A). It has been possible to observe the interrelationship of these two enzyme systems by use of a "chemical tracer", for enzyme A is active with deamino DPN whereas enzyme B is incapable of manifesting activity when this analogue is substituted for DPN. In the preceding paper, the physico-chemical properties of enzyme A were compared with a partially purified preparation of enzyme B obtained from glucose-grown bacteria. The use of deamino DPN as a "chemical tracer" in studying induced enzyme synthesis is described in this paper as are some of the properties of the induced enzyme formation.

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MATERIALS AND METHODS

Materials

Reagent grade glycerol and lactic acid were purchased from the J. T. Baker Chemical Co. 1,2-Propanediol (propylene glycol), technical grade, was purchased from the Matheson Co., Inc. Several strains of *Aerobacter aerogenes* (No. 8724, 884, 8329) and *Acetobacter suboxydans* were obtained from the American Type Culture collection. Pyridine nucleotide analogues of DPN were synthesized according to the general method of KAPLAN AND STOLZENBACH[5] except for the nicotinic acid analogue of DPN which was prepared according to the method of LAMBORG *et al.*[6].

Methods

Because of the multiplicity of enzyme activities measured, we shall refer to glycol dehydrogenase enzyme systems A and B. The enzymes will be operationally defined in terms of their enzyme activities and source as shown in Table I.

TABLE I

DEFINITION OF ENZYME SYSTEMS USED IN THE TEXT

	Growth medium	*Substrate and pyridine nucleotide requirements for enzymic activity*
Enzyme A	Glycerol plus salts	1,2-propanediol plus deamino DPN 2,3-butanediol plus deamino DPN Glycerol plus DPN 1,2-propanediol plus DPN 2,3-butanediol plus DPN
Enzyme B	Glucose plus salts	Glycerol plus DPN 1,2-propanediol plus DPN 2,3-butanediol plus DPN

Enzyme unit of activity

One unit of enzyme is defined as that amount of protein which causes an initial rate of change in O.D. (at 340 mμ) of 1.0 per min under the conditions employed. The specific activity is expressed as units/mg of protein.

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[*] The following abbreviations will be employed in this paper: DPN, diphosphopyridine nucleotide; DPNH, reduced form; TPN, triphosphopyridine nucleotide; TPNH, reduced form; RNA, ribose nucleic acid; deDPN (deamino DPN), deaminated form of DPN; AMP, adenylic acid (5′); CMP, cytidilic acid (5′); UMP, uridilic acid (5′); GMP, guanilic acid (5′); IMP, inosinic acid (5′).

[**] See MATERIALS AND METHODS for composition.

[***] See Table I for an operational definition of enzyme systems A and B.

Enzyme assay

0.5 mmole of 1,2-propanediol or glycerol and 1.2 μmoles of DPN are diluted with 2.7 ml of sodium pyrophosphate (0.1 *M*, pH 9.2). After initially determining the O.D. at 340 mμ, the reaction is started by the addition of approximately 0.2 unit of enzyme.

Growth media, methods of culture, and protein purification are described in the preceding paper.

EXPERIMENTAL

Induced enzyme formation

The results presented in the preceding paper suggest that the increase in enzymic activity observed in the case of glycerol plus DPN and 1,2-propanediol plus deamino DPN may involve the synthesis of induced enzymes. It has been possible to substantiate this viewpoint by measuring the increase in enzymic activity with time.

Procedure

A 4-l culture of a variant of *Aerobacter aerogenes* No. 8724 was grown on glucose and salts according to the method previously described. The cells (approx. $1 \cdot 10^8$/ml) were harvested in a DeLaval electrically driven centrifuge, washed once with cold water, and then centrifuged for 20 min in a Lourdes refrigerated centrifuge at maximum speed (10,000 rev./min, 16,300 × *g*). A heavy suspension of the cells was prepared by suspending the cell paste in a minimum amount of cold water (about 10–20 ml). For measuring induced enzyme formation with time, a number of 1-l Erlenmeyer flasks each containing 200 ml of glycerol media were inoculated with the same volume of cell suspension to give a Klett reading of 150 units (filter 54). This suspension was gently shaken in a water bath (30°). At intervals of time, a flask was removed from the water bath, turbidity was determined, and the suspension was chilled to 5° in a dry ice–acetone bath. The cells were removed by centrifugation, suspended in water to a final volume of 10 ml and disrupted in a Raytheon 10 kc sonic oscillator for 30 min. The cell debris was removed by centrifugation in a refrigerated International centrifuge using the high speed attachment. Protein[7] and enzymic activity were determined on the resulting crude extract.

Synthesis of induced enzyme

The synthesis of induced enzyme is easily demonstrable after a 1-h induction period in the presence of glycerol (see Fig. 1). In the absence of glycerol, no synthesis takes place. Initially, there is a lag phase of short duration before synthesis commences, but the rate of synthesis increases markedly after a 30–45 min induction period. It is also noted that the glycerol plus DPN and the 1,2-propanediol plus deamino DPN activities are elicited at the same rate during induction, suggesting that both activities are catalyzed by the same enzyme.

The constitutive activity (1,2-propanediol plus DPN) also increases during this period. This increase cannot be accounted for in terms of increase in population, for the turbidity usually remains constant during the inducing period (on two occasions

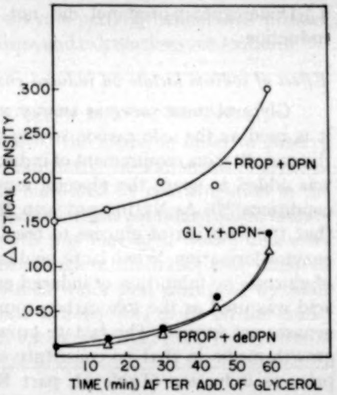

Fig. 1. Synthesis of induced enzyme. Cells were grown in a glucose-salts medium, and transferred to a glycerol-salts medium for induction. At intervals of time, aliquots of the population were removed from the media by centrifugation, suspended in cold water and disrupted by sonic vibration. The concentrations of 1,2-propanediol plus DPN (○, PROP + DPN), 1,2-propanediol plus deamino DPN (△, PROP + deDPN), and glycerol plus DPN (●, GLY + DPN) are the same as those listed in Table II. The activities recorded are the changes in O.D. at 340 mμ for the first 2 min of reaction. See the text for further details.

a 17 % increase in turbidity was noted during the inducing period) while constitutive activity increases 2–3 fold. Since the rate of increase of constitutive enzyme activity parallels the rate of formation of induced enzyme, it is suggested that the additional constitutive enzyme activity, which is manifested during the inducing period, is due to the general specificity of the induced enzyme. The results presented with lactate-grown cells are in agreement with this conclusion.

The induction period chosen for most experiments was 1 h, minimizing the possibility of spurious side effects due to contamination (sterile conditions were not maintained from the onset of induction until completion of the experiment). One relatively long-term inducing experiment (4 h) was carried out. During the course of this experiment the turbidity of the cell suspension increased 19 %, constitutive enzyme activity (1,2-propanediol plus DPN) quadrupled, and there was a 14-fold increase in the induced enzyme activity (1,2-propanediol plus deamino DPN).

Requirement for a nitrogen source during induction

Attempts were made to induce enzyme formation in resting cell suspensions (in the absence of a nitrogen source). After a 1-h induction period no induced enzyme was formed, a 64 % decrease in constitutive enzyme activity ensued, and no change in the turbidity was noted. If one assumes that the induced enzyme is being formed *de novo*, the lack of induction in the absence of a nitrogen source is not surprising since *A. aerogenes* is reputed to have no pool of free amino acids[8].

Specificity of inducing agent

The following compounds were substituted for glycerol in the inducing medium (1.5 % used in each case): *n*-propanol, glucose, 2,3-butanediol, 1,3-propanediol, 1,2-propanediol, and dihydroxyacetone. No induced enzyme was formed under these circumstances. In contrast with the inducer specificity of the β-galactosidase system[9], this enzyme induction has a strict requirement for glycerol which could not be replaced by 1,2-propanediol (the other substrate utilized by the induced enzyme) or dihydroxyacetone (the product of the reversible DPN-linked oxidation of glycerol).

2,3-Dimercapto-1-propanol did not act as inducer and did not inhibit glycerol induction.

Effect of sodium lactate on induced enzyme formation

Glycerol must serve as energy source as well as induce protein synthesis when it is used as the sole carbon source during induced enzyme formation. In studying the quantitative requirement of inducer during induction an additional carbon source was added to spare the glycerol requirement for growth (see MONOD's "gratuitous conditions"[10]). As NEIDHARDT AND MAGASANIK[11] have previously reported, we found that the addition of glucose to the inducing medium completely inhibited induced enzyme formation. When lactic acid (adjusted to pH 7.0 with NaOH) was used instead of glucose, no inhibition of induced enzyme formation was observed and when lactic acid was used as the sole carbon source in the growth media no *vic* glycol dehydrogenases are formed. The lactate growth media had the advantage over the glucose growth media in that no constitutive *vic* glycol dehydrogenase activity was present prior to induction (Table II, part B). Using lactate-grown cells one can causally relate *vic* glycol dehydrogenase activity with the addition of inducer. Table II also compares the capacity of glucose- and lactate-grown cells to induce enzyme formation when placed in an inducing medium (glycerol plus salts). It was noted that less enzyme was formed in cells grown on glucose (Table II, part A).

After induction of lactate-grown cells, each of the enzymic activities increased 8–9 fold, (*i.e.*, 1,2-propanediol plus DPN, 1,2-propanediol plus deamino DPN and glycerol plus DPN). The same relationship was found for the glucose-grown bacteria

TABLE II

EFFECT OF GROWTH MEDIA ON INDUCED ENZYME FORMATION

	Inducer	*Enzyme activity (units)**		
		Enzyme B	*Enzyme A*	
		1,2-propanediol plus DPN	*1,2-propanediol plus deamino DPN*	*Glycerol plus DPN*
A. *Cells grown on glucose*				
1. Before induction	—	0.255	0.017	0.015
2. After induction	Glycerol	0.350	0.070	0.074
Increase in enzymic activity after induction	(2/1)**	5.6***	4.1	4.9
B. *Cells grown on lactate*				
3. Before induction	—	0.039	0.029	0.057
4. After induction	Glycerol	0.327	0.262	0.471
Increase in enzymic activity after induction	(4/3)	8.4	9.0	8.3

* 500 μmoles of glycerol or 1,2-propanediol and 1 μmole of DPN or deamino DPN were diluted to a final volume of 3.0 ml with sodium pyrophosphate buffer (0.1 *M*, pH 9.3). The reaction was started with 1 unit of enzyme. The activity is recorded in terms of change in O.D. at 340 mμ per unit time.

** Increase in enzymic activity after induced enzyme formation was determined by dividing the enzymic activity after induction by the activity measured before induction except as noted in footnote ***.

*** In this case the absolute increase in activity is 0.095, which is manifested as a result of induced enzyme formation. If one assumes that the activity in the absence of inducer would be approximately 0.017 (assuming that the constitutive enzyme was not present), then 0.095/0.017 equals 5.6.

if one corrected for the initial "constitutive" enzyme activity which was present before the cells were induced (for further explanation and calculations see footnote *** of Table II).

Determination of the amount of glycerol needed for induced enzyme formation

It was not necessary to remove the lactate from the media in order to observe induced enzyme formation. In determining the quantitative measurement of glycerol needed for induction, cells grown in lactate were harvested by centrifugation in the cold and equally divided among several flasks each of which contained salts, lactate (1.5 %), and varying amounts of glycerol. Growth was measured before and after induction. After the 1 h induction period cells from each of the flasks were removed by centrifugation, resuspended in 10 ml of distilled water and disrupted by sonic vibration. The estimation of glycol dehydrogenase activity was made on the crude, clear sonicate after removing cell debris by high speed centrifugation. The results of this study are presented in Fig. 2. It can be seen that appreciable enzyme was induced when the concentration of glycerol exceeded $0.16 \cdot 10^{-2}$ *M*. In addition, the glycerol concentration required for half maximal enzyme induction was the same for the three activities measured (approx. $0.8 \cdot 10^{-2}$ *M*).

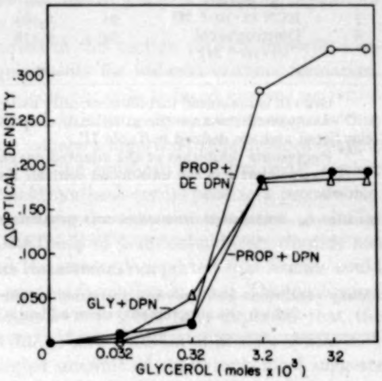

Fig. 2. Amount of glycerol needed for induced enzyme formation. Induction and assay conditions are the same as those outlined for Fig. 1 except that the growth medium contained sodium lactate as the sole carbon source for growth. See the text for other details.

Inhibition of induced enzyme formation

In addition to the inhibition of induced enzyme formation by glucose, a variety of chemical compounds was tested in an effort to learn some of the requirements for this synthesis. The effects of some of these compounds are summarized in Table III. The cells used for this study were grown in glucose-salts media and transferred to the glycerol media for induction. Expt. 1 of Table III shows the normal enzymic activity for cells derived from this source. There was a DPN-linked 1,2-propanediol dehydrogenase activity and very little activity using other glycols. Expt. 2 of this table is chosen as the control. These cells were induced for 1 h in the glycerol-salts media in the absence of glucose or any other inhibitors. Expts. 3, 4, and 5 show the results of cells which were induced in the glycerol-salts media plus various known inhibitors of purine or pyrimidine synthesis. Azaserine*, an anti-purine compound (Expt. 5) completely inhibited induced enzyme formation, and the other two compounds (thioguanine and 6-mercaptopurine) gave only slight inhibition at the concentration employed. No inhibition of the "constitutive" enzyme was noted in any of

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* Azaserine was kindly provided by Dr. MOORE of Parke-Davis and Co.

TABLE III

INHIBITION OF INDUCED ENZYME FORMATION

Expt.	*Inhibitor*	*Growth (%)**	*Enzyme activity (units)***					
			1,2-propanediol plus DPN		*1,2-propanediol plus deamino DPN*		*Glycerol plus DPN*	
			Units	*% Inhibition****	*Units*	*% Inhibition*	*Units*	*% Inhibition*
1§	—	123	0.197	—	0.012	—	0.021	—
2§	—	100	0.346	—	0.073	—	0.158	—
3	Thioguanine (80 µg/ml)	96	0.365	0	0.054	30	0.134	15
4	6-mercaptopurine (65 µg/ml)	91	0.335	7	0.054	30	0.123	25
5	Azaserine (65 µg/ml)	80	0.224	82	0.010	100	0.013	100
6	Chloramphenicol (30 µg/ml)	86	0.180	100	0.015	95	0.048	80
7	KCN ($1 \cdot 10^{-3}$ *M*)	92	0.194	100	0.022	85	0.033	93
8	Dinitrophenol ($1 \cdot 10^{-3}$ *M*)	94	0.178	100	0.008	100	0.015	100

* Growth is measured turbidometrically using the Klett-Summerson colorimeter (No. 54 filter).

** Assay conditions are the same as those listed in Table II. Units represent the enzymic activities listed and are defined in Table II.

*** Represents inhibition of the adapted enzyme formation.

§ Experiment 1 is the uninduced control, Experiment 2 is the induced control (no inhibitors added).

TABLE IV

ANTAGONISTS OF AZASERINE INHIBITION

Assay conditions and enzymic activities measured are listed in Table II. The general media to which the supplements were added is recorded in MATERIALS AND METHODS.

	Activity (units)		
	1,2-propanediol plus DPN	*1,2-propanediol plus deamino DPN*	*Glycerol plus DPN*
Control	0.379	0.317	0.384
+ Azaserine (10 µg/ml)	0.223	0.017	0.007
+ Azaserine + amino acids*	0.274	0.185	0.234
+ Azaserine + phenylalanine	0.252	0.124	0.213
+ Azaserine + tyrosine	0.236	0.133	0.208
+ Azaserine + tryptophane	0.178	0.039	0.082
+ Azaserine + glycine	0.230	0.019	0.013
+ Azaserine + lysine	0.230	0.016	0.014
+ Azaserine + arginine	0.202	0.018	0.011
+ Azaserine + AMP, CMP, GMP, UMP	0.212	0.026	0.005
+ Azaserine + AMP, CMP, GMP, UMP (80 µg/ml)	0.121	0.006	0.012
+ Azaserine + IMP	0.230	0.031	0.009
+ Azaserine + nicotinamide	0.235	0.021	0.018
+ Azaserine + histidine	0.146	0.014	0.016
+ Azaserine + glutamine	0.187	0.016	0.016
+ Azaserine + serine	0.228	0.020	0.018

* The amino acids mixture contained 10 µg/ml of each of the amino acids listed individually. The concentration of each of the separate amino acids and other compounds listed was also 10 µg/ml (except as indicated).

these experiments. Chloramphenicol, potassium cyanide, and dinitrophenol were also capable of inhibiting induced enzyme formation without affecting the constitutive enzyme. Table IV shows that the azaserine inhibition can be partially overcome by the aromatic amino acids, but not by aliphatic amino acids or ribotides. Pyrimidines, purines and ribosides are also incapable of relieving the azaserine inhibition.

DISCUSSION

In comparing the synthetic ability of glucose- and lactate-grown cells, it is found that less induced enzyme is formed in cells originally grown on the glucose-salts media (see Expt. 2, Table II). This inhibition could be due to a difference in the rate of formation of induced enzyme. That is, the inhibition might be the reflection of a difference in the capacity of glucose- and lactate-grown cells to synthesize induced enzyme. It is also possible that the inhibition is due to a small amount of glucose (or a catabolic product of glucose) remaining within the cell which is capable of partially inhibiting induced enzyme formation.

The inhibitor experiments detailed earlier in this section provide important information on the characteristics and requirements for induced enzyme formation. All of the chemicals are found to inhibit selectively only induced enzyme activities (the constitutive enzyme activity is unaffected). Inhibition by azaserine suggests that nucleic acid, or precursors of nucleic acid are somehow involved in the induced enzyme formation. The inhibition may be effected by the synthesis of "abnormal" nucleic acid or by the inhibition of normal nucleic acid synthesis (or its necessary precursors). Aromatic amino acids are capable of antagonizing the azaserine inhibition. A similar antagonism of azaserine inhibition in the synthesis of 5-aminoimidazole ribotide has been reported by GOTS AND GOLLUB[12], and HALVORSON[13] reported that leucine could relieve the azaserine inhibition of induced enzyme formation in yeast. The mechanism of this antagonism is not understood. GALE AND FOLKES have reported that the bacteriostatic effect of chloramphenicol is due to an inhibition of protein synthesis[14]. The complete inhibition caused by the higher amount of chloramphenicol suggests that the protein associated with the induced activities is synthesized *de novo* from free amino acids. Since cyanide, and dinitrophenol are known to inhibit the synthesis of high-energy compounds it is presumed that high-energy intermediates required for many synthetic reactions are also required for the formation of induced enzymes. In summary then, the requirements for induced enzyme synthesis are: the synthesis or presence of normal nucleic acid (or its precursors), amino acids (or their precursors), high energy-intermediates, and the inducer.

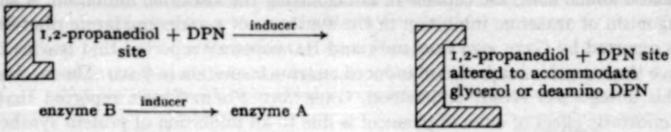
It is suggested that inducing lactate-grown cells of *A. aerogenes* with glycerol leads to the activation of a single enzyme which is capable of the oxidation of 1,2-propanediol mediated by DPN or deamino DPN and the oxidation of glycerol mediated by DPN (the same enzyme can be obtained by growing the bacterium solely on glycerol). In support of this suggestion one may site the following facts. (a) A simultaneous activation of all three enzymic activities is effected by the same concentration of glycerol. (b) The rate of formation of each of the enzymic activities is the same. (c) Chloramphenicol, azaserine, etc., inhibit the appearance of all three activities to the same extent. (d) The ratio of activities remains constant during the course of purification.

The concentration of glycerol required in this system exceeds the "saturating" concentration of inducer required for the maximal rate of production of β-galactosidase[10] (measured during the first 10 min after induction by methyl-β-D-galactoside). Since glycerol, the inducer, is metabolized by the induced enzyme, one can safely conclude that if a non-metabolizable inducer was available, the concentration required for half maximal enzyme formation would be considerably less.

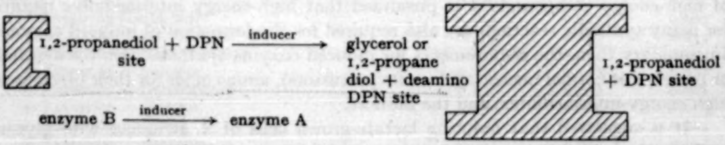
Several mechanisms can be suggested to explain the induction phenomenon resulting from the exposure of glucose grown bacteria to glycerol. It may be that enzyme B is really a partially inactive form of enzyme A which is inhibited by glucose or a catabolic product of glucose and that this metabolic inhibition is somehow relieved when the bacteria are transferred to glycerol. If this hypothesis is correct then the inhibition should be relieved when glucose grown bacteria are transferred to a medium containing glycerol plus chloramphenicol. The experimental observations contradict this hypothesis. Three alternative hypotheses may be proposed which are in agreement with the experimental results. Briefly stated these possibilities are:

1. *De novo* protein synthesis $\left\{\begin{array}{l} \text{amino acids} \xrightarrow{\text{inducer}} \text{enzyme A} \\ \text{precursor} + \text{amino acids} \xrightarrow{\text{inducer}} \text{enzyme A} \end{array}\right.$

2. Alteration of the active site resulting from the addition of inducer. In some way, the inducer initiates a change in the physical or chemical composition of the enzymically active site of the enzyme protein; this results in a glycol dehydrogenase of more general substrate and pyridine nucleotide specificity.


1,2-propanediol + DPN site
inducer
1,2-propanediol + DPN site altered to accommodate glycerol or deamino DPN
enzyme B
inducer
enzyme A

3. Synthesis (or activation) of a new site on the same protein molecule, resulting in a protein with more than one active site.


1,2-propanediol + DPN site
inducer
glycerol or 1,2-propane diol + deamino DPN site
1,2-propanediol + DPN site
enzyme B
inducer
enzyme A

The later two possibilities can be ruled out by the chloramphenicol and immunochemical experiments (presented in the preceding paper), only if it is assumed that alteration or activation of proteins does not involve some type of *de novo* protein synthesis. BRACHET and others[15–18] have presented a great deal of circumstantial evidence in favor of the hypothesis that RNA synthesis is a necessary requirement for protein synthesis. Assuming that the BRACHET hypothesis is correct, the inhibition of induced enzyme formation by azaserine supports the conclusion that the enzyme formation studied herein takes place via some form of *de novo* protein synthesis.

ACKNOWLEDGEMENTS

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A CHLOROMERCURIBENZOATE RESIN FOR THE SELECTIVE BINDING OF NONPROTEIN SULFHYDRYL COMPOUNDS

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SUMMARY

An insoluble reagent formed by the binding of sodium *p*-chloromercuribenzoate to Dowex-2 resin has been used for the selective removal of thiols from solutions or from tissue homogenates by reverse dialysis. It was possible to liberate the bound thiol from the reagent by exchange with other sulfhydryl compounds.

INTRODUCTION

Many enzyme systems require the presence of sulfhydryl groups for their activation, and often incorporate a sulfhydryl reservoir such as glutathione. Presumably,

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Abbreviations: *p*CMB, *p*-chloromercuribenzoate; EDTA, tetrasodium ethylenediamine tetraacetate.

glutathione maintains the enzyme thiol radicals in reduced form. It is at times desirable to remove or inactivate this reservoir in order to study the properties of the unprotected constituents of the system. While it is possible to dialyse low molecular weight thiols from protein preparations, other substances (not all of them known or replaceable) may be removed as well. Therefore, a method for the selective removal of thiols from biological systems seems desirable.

DAVISON AND HOFMANN have shown[1] that the addition of N-ethyl maleimide to rat adrenal tissue will arrest steroidogenesis; however, this procedure blocks the thiol groups of proteins, enzymes and glutathione indiscriminately, and therefore the results are difficult to interpret. In the course of studies on the relation of glutathione to adrenal cortical function[2], we encountered the same problem. To achieve a selective removal of soluble sulfhydryl compounds, we sought to develop an insoluble sulfhydrylbinding agent which could be used for reverse dialysis. A preliminary description of this reagent has been published[3]; subsequently we have become aware of a prior note by MILES, STADTMAN AND KIELLEY[4], describing a mercurial bakelite polymer which was prepared for much the same reasons.

EXPERIMENTAL AND RESULTS

Preparation of the reagent

Dowex-2 X 7.5 resin, 25–50 mesh (lot No. 2050, obtained from the Dow Chemical Company), was purified in the chloride form in 100-g lots by stirring mechanically for 10-min intervals with successive 200-ml portions of the following solutions: 4% NaOH (3 changes); distilled water (4 changes); 4% HCl (2 changes), distilled water (3 changes); 4% HCl (2 changes); distilled water (10 changes). The prepared resin was stored in chloride-free distilled water, which had a pH of 6.8. A weighed quantity of wet resin was placed in a small Erlenmeyer flask surmounted by a filter funnel. Into another flask was weighed a quantity of sodium *p*-chloromercuribenzoate (*p*CMB) in about 10% excess of the milliequivalents absorbable by the resin (in this case 3.1 ± 0.3/dry gram). The *p*CMB crystals were shaken with water for about 5 min, allowed to settle, and the supernatant solution poured through the filter onto the resin, which was then stirred magnetically for a minute or two. The aqueous supernatant from the resin was then poured back through a filter into the flask with the *p*CMB crystals and the process repeated over and over. It is most important to avoid contaminating the resin with *p*CMB crystals. Maximum uptake of *p*CMB was determined by testing the supernatant solution from the resin for dissolved *p*CMB. 0.5 ml of the solution was added to 0.5 ml of a glutathione standard (9.3 mg/100 ml of 2% aqueous sulfosalicylic acid) and the resulting SH concentration measured amperometrically[5]. When there was no further uptake of *p*CMB, the resin was washed 10 times with distilled water. About 90 to 92% of the theoretical amount of *p*CMB absorbable was bound to the resin.

Properties of resin–pCMB

Storage of resin–*p*CMB in distilled water at 4° for periods up to 60 days, storage for several days at room temperature, or drying of the resin had no effect on its sulfhydryl-binding properties so long as it was kept in the dark.

The uptake of cations was tested by adding approximately 2500 counts/min

^{43}KCl in water, to a small portion of resin–*p*CMB. After standing for a few minutes, the supernatant solution was decanted and the resin washed repeatedly with water. The combined washings contained 93.8% of the added radioactivity, indicating little cation retention.

Properties of the resin–pCMB–glutathione complex

1. *The effect of chelating agents*: Resin–*p*CMB reacted with glutathione in aqueous or dilute sulfosalicylic acid solutions in a manner characteristic of *p*CMB alone. The influence of chelating agents was of interest and was investigated in two ways. In one group of experiments, glutathione or [^{35}S]glutathione was dissolved in water or in 10% aqueous sulfosalicylic acid to which had been added 50 mg tetrasodium ethylenediamine tetraacetate (EDTA)/100 ml solution. This reagent was shaken mechanically with small portions of the resin–*p*CMB for 10 min and the supernatant solution examined by liquid scintillation counting[2], by amperometric titration of sulfhydryl groups, or by both methods. The removal of glutathione was essentially quantitative. In other experiments, the resin–*p*CMB was first shaken with a solution of EDTA, and the glutathione or [^{35}S]glutathione added subsequently; the same result was observed.

The effect of pH: Under the influence of varying pH, dissociation of the resin-*p*CMB glutathione complex could occur either at the resin-carboxyl bond, the mercury-carbon bond, or at the mercury-mercaptide bond. These possibilities were investigated in the following experiments. 1.0 g resin–*p*CMB was treated at neutral pH with an excess (3.257 mmoles) of [^{35}S]glutathione. The resin–*p*CMB–[^{35}S]glutathione complex was washed repeatedly with 10-ml portions of water until the 25th wash contained less than 2% of the initial radioactivity. The washings were combined and counted, and $2.08 \cdot 10^6$ counts/min (1.06 g GSH + G^{35}SH/g resin) were found to have been incorporated in the glutathione-saturated resin–*p*CMB. Solutions at integral pH's from 1 to 10 were prepared by the addition of HCl or NaOH to 0.1 *M* aqueous acetic acid. To 10-ml aliquots of these solutions, 100-mg portions of the resin–*p*CMB–[^{35}S]glutathione were added and stirred magnetically for 5 min. The supernatant solution was filtered off; two aliquots of 0.3 ml were used for measurement of radioactivity by liquid scintillation counting[2] and the remainder was reserved for the determination of mercury in duplicate by the ignition technique of FEIGL[6]. The results of two such experiments are summarized in Table I. Disruption of the

TABLE I

DISSOCIATION OF RESIN–*p*CMB–GLUTATHIONE COMPLEX AT VARIOUS pH VALUES

pH	*Liberation of ^{35}S, %*	*Liberation of mercury*	
		µmoles	*%*
1	8.3	0.01–0.02	0.01–0.03
2	11.6	0.02–0.05	0.05–0.06
3	3.5	0.5	0.63
4	3.0		
5	3.4	0.6	0.79
6	4.3		
7	5.9	0.8	0.95
8	5.2	2.5	3.2
9	6.0	5	6.3
10	2.2	$<$ 0.01	--

resin-carboxyl bond (or conceivably the mercury-carbon bonds), with the liberation of organic mercury, was detectable over the entire pH range although only microgram quantities appeared at the extreme ends of the pH scale. Maximum appearance of mercury in the aqueous phase occurred at pH 9, with 6.3 % of the mercury dissociating from the resin. The mercury-mercaptide bond behaved quite differently, showing maximum dissociation at pH 2 and 9, with a minimum at pH 3.0.

Exchange of carboxyl groups: Acetate was used as a model compound to measure carboxyl exchange at the resin-carboxyl bond. 2.0 ml of an aqueous 0.1 *M* solution of sodium [1-^{14}C]acetate was shaken with 24.6 mg resin–*p*CMB for 30 min. No removal of radioactivity from the supernatant solution was observed; therefore, the exchange of acetate-carboxyl for *p*CMB-carboxyl in the resin–*p*CMB complex appeared to be negligible. Additional evidence was secured by shaking an aqueous solution of 66.4 mg *p*CMB-glutathionate with 17.9 mg resin–*p*CMB [^{35}S]glutathionate. After 0.5 h of shaking, only 1 % of the [^{35}S]glutathione appeared in the supernatant solution and washings, indicating that little exchange occurred at the carboxyl bond under these conditions.

Stability of the mercury-mercaptide bond in the presence of thiols: 25 mg resin–*p*CMB saturated with 120,000 counts/min [^{35}S]glutathione was shaken for 30 min with 2 ml water containing 15 mg cysteine hydrochloride. 17.5 % of the [^{35}S]glutathione appeared in the washings. With 50 mg of glutathione instead of the cysteine, only 5.6 % of the [^{35}S]glutathione was washed off. However, when 1-g batches of resin–*p*CMB saturated with $4.0 \cdot 10^6$ counts/min of [^{35}S]glutathione were shaken with a solution of 1 g glutathione in 18.5 ml water adjusted to pH 6.4 with bicarbonate, from 41.2 to 53.7 % of the [^{35}S]glutathione appeared in the aqueous phase. As mentioned in previous section, *p*CMB–glutathione did not displace significant amounts of [^{35}S]glutathione from the resin.

Removal of tissue thiols by reverse dialysis

Deproteinized tissue extracts: 10 g of fresh rat liver was homogenized in a micro Waring blendor in cold 10 % sulfosalicylic acid, made up to 100 ml volume, and filtered after standing for 1 h. 8 g of resin–*p*CMB were enclosed in a bag of 1 cm wide Visking dialysis casing, with the air displaced by about 15 ml of the liver filtrate. The bag was placed in a 2.5 cm diameter glass cylinder which was partially filled with 45 ml of filtrate, stoppered, and oscillated mechanically 30 times/min. At hourly intervals, 1.0-ml samples of filtrate were removed from the cylinder and the sulfhydryl concentration measured amperometrically. (The stability of resin–*p*CMB in the presence of sulfosalicylic acid was demonstrated in the model experiments described above.) By 4 h, there had been a 40 % removal of sulfhydryl, and by 6 h a total of 93 %.

Tissue incubations. In these studies both rat-liver homogenate and cow adrenal whole-cell homogenate in KREBS-RINGER-phosphate were studied using a standardized technique for tissue incubation. The dialysis bags consisted of 4 cm lengths of 1 cm wide Visking casing. 0.1 g of resin–*p*CMB was placed in the bags and air displaced by adding filtrate from a sample of boiled homogenate. 1 ml of homogenate or brei, 114,000 counts/min of [^{35}S]glutathione and a resin bag were placed in a 15 × 60 mm vial closed with a plastic-lined screw cap. A number of these vials were inserted end to end within lengths of 20 mm diameter glass tubing which were maintained at 37°

and rotated at 13 rev./min. Vials were removed at intervals, aliquots of the homogenate precipitated with sulfosalicylic acid and 0.3-ml portions of the supernatant solution taken for liquid scintillation counting. The results of experiments with adrenal whole-cell homogenates are shown in Table II. Several experiments with liver homogenates were comparable.

TABLE II

REMOVAL OF THIOLS FROM ADRENAL HOMOGENATES

Reaction time	*No. of Expts.*	*$G^{35}SH$ removed, %*
0	2	0
30	4	24.0
60	5	33.0
90	20	62.2

DISCUSSION

The studies indicate that the combination of sodium *p*CMB with Dowex-2 forms an insoluble and relatively stable reagent, which can be used successfully for the removal of low molecular weight sulfhydryl compounds from solutions or tissue suspensions by reverse dialysis. In experiments to be reported elsewhere, it has been found that removal of a sufficient fraction of sulfhydryl from adrenal whole-cell homogenates results in cessation of steroidogenesis, but that this effect can be prevented if the homogenate is protected with added glutathione. This is evidence of the selective action of the resin–*p*CMB reagent for sulfhydryl groups and implies that exchange of tissue constituents with *p*CMB on the resin did not occur measurably in this system. Whether this would obtain in systems with very high concentrations of anions such as phosphate, sulfate, nitrate, etc. or whether these ions would cause release of the *p*CMB by the resin would have to be determined in the individual systems. In biologic as contrasted to purely chemical systems, return of biologic activity by restoration of the sulfhydryl compounds must be demonstrated before the selective action of the reagent can be accepted. The relative ease with which bound glutathione can be removed from the resin–*p*CMB reagent by exchange with added thiol (particularly if a volatile thiol or H_2S can be added instead of cysteine) suggests that the reagent might be of value for concentrating or isolating thiols in circumstances where classical procedures such as formation of copper or cadmium mercaptides are not feasible.

ACKNOWLEDGEMENT

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NUCLEIC ACID METABOLISM IN A SLIME MOLD WITH SYNCHRONOUS MITOSIS*

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SUMMARY

In the synchronously dividing slime mold, *Physarum polycephalum*, the synthesis of DNA as shown by incorporation of ^{14}C from [6-^{14}C]orotic acid into DNA-thymine, occurred immediately after the nuclear division and lasted for 1–2 h. During the remainder of the interphase, which might last from 12–20 h, there was no significant labeling of the DNA. The incorporation of label into RNA at any time of the cell cycle suggested that RNA synthesis was continuous, although there was some indication that this process might be retarded at the time of division and DNA synthesis. The relative timing of DNA synthesis and nuclear division would tend to rule out the synthesis of DNA as a "trigger" for mitosis. Preliminary experiments indicated that the mold was capable of converting orotic acid to thymine nucleotides throughout the interphase; this suggests that the control of DNA synthesis must occur at some point very close to the final polymerization of the molecule.

INTRODUCTION

The desire to study reactions which are related to the division cycle of the cell has prompted many investigators to develop biological systems with artificially induced synchrony[2]. A few systems have been found in which synchronous division occurs spontaneously, the best known being the cleavage of the fertilized egg[3] and the formation of microspores in certain plants[4]. In the slime mold *Physarum polycephalum*, nuclear division is synchronous in relatively large plasmodia. The occurrence of spontaneously synchronous mitoses in this myxomycete was first reported by HOWARD[5]. More recently, GÜTTES *et al.*[6] have made an extensive study of the morphology of this organism and have described in detail the various stages of its life cycle.

The present report concerns the determination of the temporal relationship

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Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; DNA-P and RNA-P phosphorus in deoxyribo- and in ribo-nucleic acid respectively; DNA-thymine, thymine in deoxyribonucleic acid; RNA-uracil, uracil in ribonucleic acid.

[*] A preliminary report was presented at the annual meeting of the Federation of American Societies for Experimental Biology, Chicago (U.S.A.), 1957[1].

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between synthesis of DNA and mitotic nuclear division in the vegetative stage of *Physarum polycephalum*. For this purpose the conversion of [6-^{14}C]orotic acid to DNA-thymine was studied in relation to the cycle of plasmodial growth. In addition, the conversion of orotic acid to thymine derivatives of the acid-soluble fraction as well as to RNA-uracil was also determined.

METHODS

Cultures

The mold was grown on a soluble medium as described by DANIEL AND RUSCH[7]. [6-^{14}C]orotic acid was used as radioactive precursor and was added to the medium prior to autoclaving whenever radioactive medium was prepared. Unless otherwise stated, the amount of radioactive orotic acid was 0.3 μC/ml with specific activity 1 μC/μmole.

To facilitate transfer and removal of samples, the molds were grown in 9-cm Petri dishes as shown in Fig. 1. The plasmodium was supported by a piece of filter paper (Whatman No. 40), which in turn was supported by a layer of glass beads, 4 mm in diameter. The addition of approximately 15 ml of medium was sufficient to bring the liquid to the level of the filter paper; thus the upper surface of the mold was exposed to air. Several small perforations were put in the filter paper to prevent the trapping of air bubbles below the paper, which would have reduced the contact between paper and liquid. The Petri dishes with beads and filter paper were autoclaved prior to addition of the medium and the mold. Aseptic conditions were maintained during the transfers as well as during the removal of the samples.

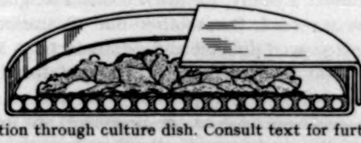

Fig. 1. Section through culture dish. Consult text for further details.

Stock cultures of the mold were grown as shake cultures, as described by DANIEL AND RUSCH[7]. To convert the mold to a surface culture, the shake suspension was centrifuged at about 250 × g, the old medium discarded, and a 1-ml aliquot of the mold-sediment transferred to a sterilized Petri dish containing beads and filter paper. The mold was left for 6–12 h on the moist paper before medium was added to the Petri dish. This "starvation period" was found to facilitate the fusion of the mold fragments. After about 3 days the cultures were ready for the final transfer. Pieces of filter paper, 1–2 cm^2 in size, were cut along the edge of the mold outgrowth and transferred to Petri dishes complete with filter paper and medium. After a growth period of 2 days the plasmodia were approximately 4–5 cm in diameter and were ready for the experiments.

About 30 cultures were usually prepared for an experiment. To determine the stage of the cultures within the division cycle, samples for smears were taken every hour with a pointed scalpel. The smears were fixed in alcohol and studied under a phase contrast microscope[6], at a magnification of 1000 times. The first 8–10 cultures that were found to be in mitosis when sampled were selected for the subsequent work. The frequency of sampling was increased whenever a mold was found to be in prophase. By a comparison of the smears with a previously established time schedule for the various mitotic stages of this mold[6], the time of division—*i.e.*, the separation of the daughter nuclei at the end of anaphase—could be estimated with an error of less than 5 min.

For studying the incorporation of the precursor, filter paper and mold were transferred at the preselected time to a new Petri dish containing radioactive medium. Samples for chemical analyses and radioactivity measurements were obtained by cutting off and removing a part of the filter paper with adhering piece of mold, while the remainder of the mold was saved for further sampling and analyses. In this way each mold could be used for serial analyses or be made to serve as its own control.

Analytical procedures

Each piece of mold to be analyzed was homogenized in a Potter-Elvehjem homogenizer for approximately 20 sec with 4 ml of ice-cold 5 % trichloroacetic acid (TCA). The entire homogenate was transferred to a 15 ml ignition tube in which all subsequent steps were carried out. The sample was centrifuged and the sediment washed, consecutively, with 5 % TCA acid (twice), 60 % ethanol, 95 % ethanol (twice), and finally with acetone. After all samples were dried in a vacuum desiccator, their net weights were determined. Usually, the dried residues weighed 10–30 mg. With the relatively small samples involved, the isolation and separation of the nucleic acids by conventional methods proved difficult and unsatisfactory. We therefore used the method introduced by JERVELL *et al.*[8] and hydrolyzed the dried residues directly with 98 % formic acid in sealed tubes kept at 165° for 2 h.

Since [6-^{14}C]orotic acid was used as the precursor, we were interested only in the pyrimidine bases of the nucleic acids. Furthermore, since thymine and uracil are unique to DNA and RNA, respectively, the isolation of these two pyrimidine bases or their derivatives from the "acid-insoluble" material should provide a relative measure of the radioactivity as well as of the quantities of each of the two nucleic acids. With the above method for hydrolysis, DNA-thymine is liberated as the free base, whereas RNA-uracil is obtained as a mixture of uracil and uridine, with the latter component predominating. These three compounds were isolated by a combination of ion exchange and paper chromatography as previously described by JERVELL *et al.*[8]. The specific activities of the DNA-thymine and RNA-uracil were determined on the chromatographically isolated thymine and uridine, respectively. Aliquots were plated on aluminum planchets and the radioactivity measured in a gas-flow proportional counter. The amounts of the isolated compounds were determined spectrophotometrically.

In a few cases the amounts of DNA and RNA in the mold samples were determined by more direct methods. Deoxyribose was measured by a procedure described by KECK[9], and ribose by the orcinol method[10]. Assuming the ratio of purines/pyrimidines to be 1.0 in the nucleic acids of the mold, the following molar ratios were calculated for the isolated components: thymine/DNA-P = 0.26 and [uridine + uracil]/RNA-P = 0.21. A relatively good agreement among the analyses from different samples indicated that the measurement of the isolated pyrimidine derivatives could serve as a means of estimating the total amounts of nucleic acids in the samples*.

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* Representative values for the content of nucleic acids in the mold were 190 μatoms RNA-P and 15–30 μatoms DNA-P/g of dried mold residue.

A partial fractionation of the acid-soluble material to yield thymine of nucleotide and non-nucleotide origin was carried out in one experiment. The molds in this case were homogenized with 5 ml of ice-cold 0.3 *N* perchloric acid and the insoluble material washed with a second 5-ml portion of the acid. One volume of ethanol was added to the combined extracts to precipitate mucous material. Following the addition of 0.2 ml of 0.25 *M* Na_2SO_4 to each sample, the solutions were neutralized with KOH and back-titrated with HCl to give a reaction slightly acid to phenol red. The addition of Na_2SO_4 served to remove calcium ions which had been carried along from the medium and which had been found to form precipitates with organic phosphate esters under the prevailing conditions. Calcium sulfate and potassium perchlorate were removed by centrifugation in the cold, and the neutral supernatant solution was added through a 3-ml column of Dowex 50-X8 (hydrogen form) onto a 3-ml column of Dowex 1-X10 (formate form). The columns were washed with 20 ml of 0.01 *M* acetic acid, and the effluent and wash were collected as "fraction A." This fraction should contain, among other compounds, any thymidine and free thymine from the mold. Subsequent elution of the Dowex 1 column with 30 ml of 1.0 *N* HCl yielded a "fraction B". It had previously been established that this latter fraction would contain orotic acid as well as any phosphorylated thymidine derivatives present in the original perchloric acid extract.

All fractions "A" and "B" were evaporated to dryness *in vacuo*, and the residues were hydrolyzed with 98 % formic acid and carried through the subsequent steps for isolation of thymine, following the procedure given above for the acid-insoluble materials. Since in this particular experiment we were interested only in determining the total ^{14}C present in the thymine derivatives of the two fractions, carrier thymine was added to the samples before the paper chromatography to facilitate location of the compound. The isolated thymine spots were eluted and their content of radioactivity determined as described above.

RESULTS

DNA synthesis

To obtain a preliminary estimate of the period of DNA synthesis, individual plasmodia were transferred to radioactive medium at different times following nuclear division. After 3 hours' exposure to the radioactive medium, one half of each mold was harvested for analysis and the other half sampled periodically to determine the time of the next division. In the upper frame of Fig. 2 the heights of the columns show the specific activity of the DNA-thymine obtained from the various molds. The width of each column indicates the period during which the particular mold was exposed to the precursor. The time axis has been adjusted to an interdivision time of 18.6 h, which was the average found in this group. It can be seen that the most active incorporation of ^{14}C from orotic acid into DNA-thymine occurred very close to the time of division. The columns in the lower frame of Fig. 2 represent the specific activities of RNA-uracil obtained from the same molds. The incorporation of ^{14}C into the RNA-uracil in all of the samples demonstrated that the virtual absence of labeling of the DNA-thymine during most of interphase was *not* caused by failure of the mold to take up the precursor from the medium.

It was clearly desirable to time the period of the labeling of DNA more precisely.

Since a shortening of the period of exposure to radioactive medium would lead to less efficient incorporation, a different approach was tried. As already indicated from the previous experiment, essentially no labeling of DNA occurred during the greater part of the interphase. Leaving the mold exposed to radioactive medium at a time

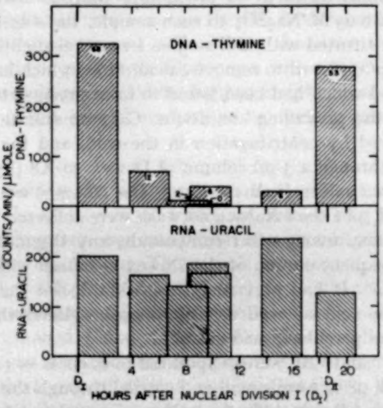

Fig. 2. Incorporation of ^{14}C into pyrimidines of nucleic acids after 3 hours' exposure of culture to [6-^{14}C]orotic acid. The columns represent in the upper frame the DNA-thymine and in the lower frame the RNA-uracil. The numbers on the columns for the DNA-thymine indicate the total radioactivity in counts/min, recovered in this compound from the entire sample. The radioactivity of the medium in this particular experiment was 0.2 μC of ^{14}C/ml. The time axis in this graph was adjusted to an average inter-division time of 18.6 h. In this and in the subsequent graphs the time of nuclear division is defined as the time of the separation of the daughter nuclei at the end of anaphase.

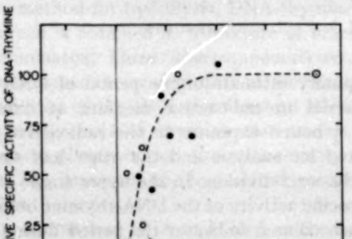

Fig. 3. Incorporation of ^{14}C from [6-^{14}C]orotic acid into DNA-thymine in relation to the time of nuclear division. The cultures were transferred to radioactive medium approximately 4 h after the previous division and left in this medium until the time of sampling. The data have been corrected for individual differences among the cultures by expressing the specific activity of each sample as % of the specific activity of the second half of the same plasmodium, which was left in the medium until 4 h after division. The specific activity of the DNA-thymine in these latter samples averaged about 1600 counts/min/μmole. The open and closed circles indicate the values obtained with two different groups of mold cultures.

when there is no synthesis of DNA would nevertheless permit the uptake of radioactive orotic acid by the mold and might also lead to the production of some more immediate radioactive precursor of the DNA molecule. In this way the efficiency of the incorporation of radioactivity into DNA, when synthesis does occur, might be greatly increased. The analysis of molds harvested during the period when DNA was presumed to be synthesized should then yield information as to the exact onset and duration of this process.

Fig. 3 shows the data obtained from two groups of molds which were transferred to radioactive medium approximately 4 h after nuclear division (division I), and allowed to grow in this medium until the final sampling, One half of each mold was sampled at a preselected time close to the next division (division II); the other half in all cases was left in the radioactive medium until 4 h after division II so that it would complete its synthesis of DNA. In each case the specific activity of the DNA-thymine from the first half of the mold was expressed as a percentage of the value obtained from the second half; this served as a means of correcting for individual differences among the cultures. The graph (Fig. 3) clearly shows that there was no significant incorporation of ^{14}C into DNA prior to the nuclear division II. Shortly after the division there was a rapid uptake of radioactivity, and a maximum level of labeling appeared to be reached between 1 and 2 h after mitosis*.

Obtaining a pure compound rather than a quantitative recovery was emphasized in the isolation of DNA-thymine. It was found, however, that the DNA-thymine per unit dry wt. increased greatly during the period of ^{14}C incorporation. The maximum value observed was 172 % of the premitotic value. If we assume that the dry wt. of the mold increased at a normal rate during the period of DNA synthesis, the reported value would suggest that the DNA content was indeed doubled during the period of ^{14}C-incorporation, indicating further that the labeling was a result of *net* DNA synthesis.

RNA synthesis

In all of the cultures there was extensive incorporation of ^{14}C from orotic acid into the uracil moiety of the RNA. To investigate the course of the RNA labeling, a group of molds was transferred to radioactive medium approximately 4 h after division, and 4–5 consecutive samples were taken for determination of specific activity of the RNA-uracil. Multiple samplings were possible in this case, since the greater RNA content of the mold permitted the use of smaller samples for analysis. The results from this experiment are plotted in Fig. 4. In agreement with the preliminary observations, recorded in Fig. 2, there was a continuous labeling of RNA during interphase, at a time when no DNA synthesis occurred. The apparent break in the curves at the time of division and of DNA synthesis is another interesting feature of the data.

Acid-soluble thymine derivatives

The conversion of orotic acid to DNA-thymine involves a series of intermediate steps[11] and it became of interest to test whether some of the more immediate pre-

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* Similar data (unpublished) have been obtained by the use of [8-^{14}C]adenine or [2-^{14}C]-thymidine as precursor and by the separation of the DNA from RNA after the addition of carrier DNA.

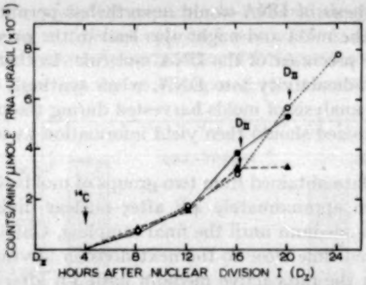

Fig. 4. Incorporation of ^{14}C from [6-^{14}C]orotic acid into RNA-uracil in relation to the time of nuclear division. The cultures were transferred to radioactive medium approximately 4 h after the first division (D$_{I}$) as indicated by the arrow (^{14}C). Each curve indicates the results obtained in a single plasmodium. Since the inter-division periods were different in these cultures the second division (D$_{II}$) has in each case been indicated by an arrow.

cursors of the DNA would be formed only at the time of DNA synthesis, or whether their production would occur in other periods of the cell cycle as well. Three cultures were transferred to radioactive medium 4 h after division. One half of each plasmodium was harvested 4.5 h later, at which time the molds were in mid-interphase. The second half was collected in late prophase, which for these particular cultures occurred 10–12 h after the transfer to radioactive medium. As it was believed that the "pool size" of the individual thymine derivatives would be quite small, isolation of the individual compounds was not attempted. Instead, an estimate was made of the amount of radioactivity in the nucleotide and non-nucleotide thymine derivatives in the acid-soluble fraction as described above. The results of this fractionation are shown in Table I. It should be noted that thymine nucleotides were formed from orotic acid in mid-interphase, as judged from the radioactivity recovered in the nucleotide-thymine fraction (fraction "B"). There was very little difference between the values found at mid-interphase and in late prophase when the data were expressed

TABLE I

CONVERSION OF [6-^{14}C]OROTIC ACID TO THYMINE DERIVATIVES

			Acid-soluble		*Acid-insoluble*	
Time of sampling	*Exposure to [6-^{14}C]orotic acid* (h)*	*Mold No.*	*Fraction A non-nucleotide thymine counts/min***	*Fraction B nucleotide thymine (counts/min)*	*DNA-thymine (counts/min)*	*Estimated final DNA-thymine (counts/min)*
Mid-interphase	4.5	I	47	376	14	(800)
	4.5	II	131	687	62	
	4.5	III	99	527	26	
Late prophase	11	I	164	418	40	
	10	II	74	762	37	
	12	III	114	535	67	

* 0.5 μC/ml of medium (1.9 μC/μmole).

** All of the radioactivity data represent total activity calculated for a sample of mold containing 1.0 μmole RNA-uracil or approximately 25 mg of dry wt. material.

as total radioactivity in a sample of standardized size. In the period between the two samplings, however, the plasmodia were increasing in mass, so that on a "per nucleus" basis there was an increase in the thymine nucleotide level, estimated to be about 35 %*.

The small amount of radioactivity recovered from the acid-insoluble thymine fraction (DNA-thymine) was probably a result of contamination by the acid-soluble thymine derivatives. The data clearly demonstrate that the radioactivity of the acid-soluble thymine could not be the result of a partial hydrolysis of radioactive DNA during the fractionation of the samples. The last value in the table represents an estimate of the radioactivity to be found in the DNA if the cultures had been permitted to go through with the division and subsequent DNA synthesis. This value, which is an average from a series of other cultures grown in the same medium, is of the same magnitude as the total radioactivity found in the nucleotide-thymine fraction. It appears, therefore, that in this organism the precursors of DNA-thymine, at least up to the thymidylic acid stage, are available much earlier than the onset of the DNA synthesis.

DISCUSSION

From the data reported it seems clear that in the synchronously dividing slime mold, ***Physarum polycephalum***, synthesis of DNA takes place during a very short part of the cell cycle. The duration of the synthesis in a single nucleus may possibly be shorter than indicated in Fig. 3, since a less rigid synchrony of DNA synthesis than that of division would tend to increase the over-all period of incorporation. The time of the period of synthesis in relation to mitosis is somewhat unexpected since, for most materials studied, the synthesis of DNA occurs during the latter part of interphase. DNA synthesis in telophase or in early interphase is, however, not unique and has been reported earlier[12]. In the present case we are dealing with an organism which has an extremely short period between mitosis and synthesis of DNA and a long period between synthesis of DNA and the next division—*i.e.*, a short G_1 and a long G_2 in the terminology of HOWARD AND PELC[13].

The low level of radioactivity found in the DNA isolated during the greater part of interphase was possibly a result of contamination by the acid-soluble fraction (*cf.* Table I). Although this explanation still needs to be proved, the present data can at least rule out the possibility of any extensive exchange of DNA material in the absence of DNA synthesis.

The timing of the synthesis of the DNA in this case renders it unlikely that synthesis of DNA *per se* should be a "trigger" for nuclear division, however necessary the duplication of DNA is for further mitotic division of the mold nuclei. The extremely close synchrony in the division of the nuclei further suggests that the "triggering principle", if such exists, either originates in the cytoplasm or can be transmitted to the other nuclei through the cytoplasm.

As to what "triggers" the DNA synthesis, we have very little information. It is known that several enzymic steps, not required for other functions of the cell, are involved in the synthesis of DNA[11]. Among these are the conversion of riboside derivatives to the corresponding deoxyriboside derivatives, the methylation of deoxyuridylic acid to thymidylic acid, the formation of deoxyriboside triphosphates, and the final condensation of these to DNA polymer. Any one of these steps could *a priori* be considered as a possible point of control for DNA synthesis.

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* In a parallel experiment (unpublished) the conversion of [2-^{14}C]thymidine to thymine nucleotides was also shown to occur during interphase. In this case the total radioactivity of the thymine nucleotides in late prophase was on the average 30 % higher than that at mid-interphase.

Our data on the production of thymidine nucleotides in the absence of DNA synthesis bear directly on this question. Although our present fractionation procedure does not permit any conclusion as to the original phosphorylation level of the thymine nucleotides, the data indicate that the mold retained its ability to convert orotic acid to at least thymidylic acid throughout the cell cycle. Thus, the synthesis of DNA is probably controlled at a point subsequent to the formation of deoxymononucleotides. The question of whether the ultimate control is at the point of the deoxynucleotide kinase or the DNA-polymerase[14], or whether it depends instead on the physical state of the pre-existing DNA (*e.g.*, the availability of a template surface), should now be open for experimental attack.

The apparent break in the incorporation of ^{14}C from orotic acid into the RNA, as indicated by the curves in Fig. 4, may imply depression of RNA synthesis at the time of division and DNA synthesis. It is, at present, impossible to say whether this phenomenon is related to the division or whether a competitive situation exists between the synthesis of RNA and the synthesis of DNA. Results obtained by other investigators[15,16] indicate that the latter explanation is the more likely one.

ACKNOWLEDGEMENT

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EFFECTS OF HALOGENATED PYRIMIDINES ON THE GROWTH OF TOBACCO MOSAIC VIRUS

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SUMMARY

The inhibition of TMV formation and the incorporation of 5-fluorouracil into viral RNA occurs in *Phlox drummondi* as well as in the Turkish tobacco host. The inhibitory action of 5-fluorouracil on the formation of TMV in isolated leaf disks of Turkish tobacco plants is dependent on the time interval which has elapsed between infection and exposure to 5-fluorouracil. 5-fluoroorotic acid, 5-fluorouridine, and 2-thio-5-fluorouracil are also inhibitors of the formation of TMV, and the first two compounds also lead to the formation of viral nucleic acid which contains 5-fluorouracil. Uridine will reverse the action of 5-fluorouracil, while thymidine is without effect. Halogenated pyrimidines, which inhibit the formation of DNA in other systems, have little effect on the formation of TMV.

INTRODUCTION

The halogenated pyrimidine, 5-fluorouracil (FU), has been shown to be an inhibitor of growth in diverse biological systems[1] including the growth of phage T2 in *Escherichia coli*[2], and the formation of tobacco mosaic virus (TMV) in cultured Turkish tobacco leaves[3,4]. In a previous study[5] it was shown that the inhibition of the formation of TMV was accompanied by the replacement of up to 47 % of the uracil of the virus ribonucleic acid (RNA) by fluorouracil. The infectivity of the FU containing virus was identical to that of normal virus when assayed on several host plants in which tobacco mosaic virus gives rise to local lesions. In Turkish tobacco plants, where the virus gives a systemic infection, the rate of virus growth following infection by the FU containing virus was smaller than the corresponding rate following infection by the normal virus.

The mechanisms of the inhibitory action of FU and some of its derivatives have been studied in various bacterial and mammalian systems[6-9]. The present paper is concerned with attempts to elucidate the mechanism of the inhibition of TMV formation by FU. A correlation of the yield of virus with structural variations in the FU molecule gave some idea as to the sites of action of FU. A study of a few of the variables involved in the growth of TMV in the presence of fluorouracil has yielded

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information about the early stages of the biosynthesis of the virus. In addition the action of FU on the ribonucleic acid metabolism in uninfected leaves was studied[*].

EXPERIMENTAL

Materials

The 5-fluoropyrimidine derivatives were a gift from Dr. R. DUSCHINSKY, Hoffmann-LaRoche, Inc., Nutley, New Jersey. The 5-bromouracil was a gift from the Sigma Chemical Company, St. Louis, Missouri. The 5-chlorouracil was prepared by Dr. G. RUSHIZKY of these laboratories, and the 5-iodouracil was obtained from Dr. G. HITCHINGS, Burroughs Wellcome, Inc., Tuckahoe, New York.

Assays and culture techniques

Except where otherwise noted, leaves of Turkish tobacco plants were infected with TMV, and after 18 h the leaves were removed from the plants. Disks were punched from the leaves and floated on aqueous solutions of the materials to be tested. After 7–8 days of incubation the disks were washed and frozen, and the virus was isolated. Details of these procedures are given in a report[5].

RESULTS

Effect of FU on the yield of TMV under various conditions

The effect of increasing concentrations of FU on the amount of TMV produced in excised disks of Turkish tobacco leaves has been described[5]. These results demonstrated that incubation of infected leaf disks on a 0.1 % solution of FU decreased the yield of virus by about 50 % (Table I) and, furthermore, some of the uracil of the virus was replaced by FU. The following experiment was carried out to ascertain whether this inhibition and concomitant incorporation of FU took place in a different, unrelated host for TMV production. For this purpose leaves of plants of *Phlox drummondi* (Burpee's Giant Tetra Red, W. A. Burpee Company, Riverside, California) two months old were infected with a 100 μg/ml solution of TMV in 0.1 *M* sodium phosphate buffer, pH 7.0. After 18 h equal weights of infected leaf segments were floated either

TABLE I

EFFECTS OF 5-FLUOROURACIL ON THE FORMATION OF TMV IN TWO DIFFERENT HOSTS[*]

Host plant[**]	*Inhibition by fluorouracil Virus yield-percent of water control*	*Fluorouracil content of virus RNA Percent of normal uracil content*
Turkish tobacco	50 ± 10	28–47[***]
Phlox drummondi	57 ± 10	38

[*] Half leaves were cultured either on 0.1% FU solution or water for 7 days.

[**] In the case of both species about 50% of the FU was absorbed by the leaf segments from the culture medium.

[***] Extreme range of values observed in several experiments.

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[*] The following abbreviations are used in this manuscript: 5-fluorouracil, FU; tobacco mosaic virus, TMV; ribonucleic acid, RNA; deoxyribonucleic acid, DNA.

on water or on a 0.1 % solution of FU. The leaves were incubated, and the virus was isolated as previously described for tobacco leaves[5]. It was found that FU causes similar reductions in the yield of virus in leaves of *Phlox drummondi* and Turkish tobacco. Moreover, FU was incorporated into the viral RNA in both of these unrelated plant hosts (Table I).

In order to ascertain the effect of exposure to FU at various time intervals after infection, disks were punched from Turkish leaves and floated on water 6 h after infection of the leaves. At the various times indicated in Fig. 1, the water in three dishes was replaced by a 0.1% solution of FU. All of the disks exposed to the FU solution were incubated for a total of eight days after infection. The disks were then harvested, and the amount of virus produced was determined. The average amount of virus produced in each set of three dishes is expressed as the percent of the virus yield in a water control that was incubated for eight days. The results are plotted in Fig. 1 as the curve labeled "Fluorouracil Treated". These results show that when the disks are exposed to a 0.1 % solution of FU at times immediately after infection (as the 1/4 and 1 day points in Fig. 1) and then incubated in the presence of FU for a total period of eight days subsequent to infection, the yield of virus is markedly reduced. On the other hand, if the disks are incubated on water for several days and then exposed to the FU solution for the remainder of the eight day incubation period (for example, the 5 and 6 day points in Fig. 1), the yield of virus as compared to that of the water control is not substantially reduced. In this experiment it was also important to determine how much virus was present in the leaf disks at the time of exposure to the FU solution. For this purpose three dishes of leaf disks, that had been incubated on water, were harvested each time another set of disks was exposed to the FU solution. The average amount of virus present in each set of dishes that had been incubated on water was expressed as the percent of the amount of virus present in the water control that had been incubated for eight days. These results are plotted in Fig. 1 as the curve labeled "Growth Curve". It is important to note that the points plotted as the "Growth Curve" refer to disks which had been incubated only on water, and the total time of incubation of these disks after infection varied

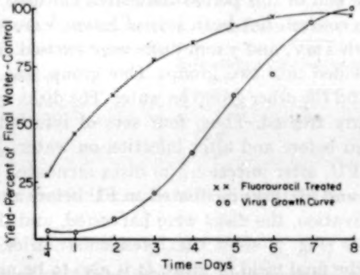

Fig. 1. Yield of TMV as a function of the time of addition of FU in days. Turkish tobacco leaves were infected at 0 time. At 6-h disks were punched from the leaves and floated in water. At the times indicated by the X designation, 3 sets of leaf disks were placed in a 0.1 % solution of FU. The disks exposed to the FU solution were all harvested after 8 days. At the same time a growth curve was determined by harvesting infected disks that had been incubated on water for periods of time indicated by the o designation.

from 1/4 to 8 days. The points plotted as the "Fluorouracil Treated" curve refer to disks which were exposed to both water and FU solution for varying periods of time, but the total time of incubation after infection in this case was eight days. The results of this experiment, as shown in Fig. 1, indicate that exposure of infected disks to a 0.1 % solution of FU at times shortly after infection results in a definite decrease in the final yield of virus. The effect of exposure to FU is greatest at the times when the virus content of the disks is small.

The effect of incubation of leaves with FU prior to infection with TMV was ascertained by culturing leaves of Turkish tobacco plants on water or a 0.1 % solution of FU for 7 days. At the end of this period the leaves cultured on FU were not quite as green as the water control, but both sets of leaves were still turgid. The leaves were then infected with TMV, and 7 mm disks were excised. The disks from the FU grown leaves were divided into two groups. One group was again cultivated on a 0.1 % solution of FU and the other group on water. The disks from the leaves cultured on water were similarly treated. Thus, four sets of infected disks were cultured; *i.e.*, (a) disks incubated before and after infection on water; (b) disks incubated on water before, and on FU, after infection; (c) disks incubated on FU before, and on water after, infection; and (d) disks incubated on FU before and after infection. After five more days of cultivation, the disks were harvested, and the yields of TMV were determined. The results (Fig. 2) show that pre-incubation of leaves with FU has a pronounced effect on the final yield of virus. It is also to be noted that the effects of pre-incubation on FU are not reversed by placing the infected plant tissues on water.

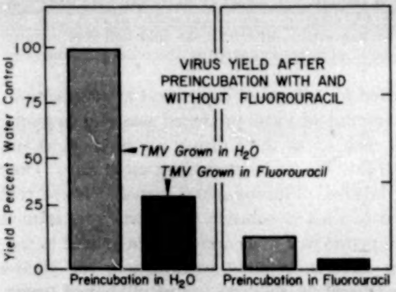

Fig. 2. Inhibition of virus formation by FU under various conditions. Half leaves of Turkish tobacco were cultured on water or FU for one week, infected, and 7-mm disks were excised. An equal number of disks from each set of leaves was then floated on water or 0.1 % FU and cultured for 5 days. The amount of virus formed after 5 days was determined.

Effect of FU on the ribonucleic acid metabolism of normal leaves

The effect of FU on the metabolism of the various leaf ribonucleic acid fractions was investigated in the following experiment. Corresponding quarter leaves were incubated either in water or in a 0.1 % solution of FU for 7 days. Then *ca.* 36 μC of ^{32}P (sodium phosphate, pH 7) was added to each set, and incubation was continued for an additional 24 h. The leaves were then thoroughly washed with water and ground with sand in the presence of an equal wt. of sucrose-phosphate buffer (0.25 *M* sucrose–0.02 *M* phosphate buffer pH 6.8). The different cell fractions were separated by differential centrifugation according to the procedure of ZAMECNIK[10]. Each fraction was purified by one additional cycle of differential centrifugation, but some contamination of the mitochondrial and microsomal fractions by chloroplast fragments was unavoidable in view of the fragility of tobacco leaf chloroplasts. The RNA was isolated by the hot salt extraction procedure of DAVIDSON AND SMELLIE[11]. The radioactivity of known amounts of RNA was determined, and it was found that incubation in a 0.1 % solution of FU resulted in a 3–5 fold decrease in the incorporation of inorganic phosphate into the RNA of various subcellular fractions (Table II).

TABLE II

EFFECT OF 5-FLUOROURACIL ON PHOSPHATE METABOLISM OF SUBCELLULAR FRACTIONS

Cellular fraction	*Radioactivity of RNA (counts/min/mg)*	
	Control	*5-Fluorouracil grown*
Chloroplasts	1400	280
Mitochondria	240	92
Microsomes	24	8.8

Possible incorporation of FU into chloroplast RNA

Since a large amount of moderately radioactive nucleic acid was obtained from the above chloroplast fraction, the chloroplast nucleic acid was degraded with alkali, and the resulting nucleotides were submitted to electrophoresis at pH 9.2. Radioautography revealed the presence of a band of radioactive material which migrated with the velocity of a fluorouridylic acid. The amount of this rapidly moving material was too small to be observed under u.v. light, and, consequently, no definite identification was possible by means of its u.v. absorption spectrum. Attempts to demonstrate the incorporation of FU into the RNA of separated tips of Turkish tobacco plants by culture in the presence of FU and ^{32}P labeled inorganic phosphate were inconclusive. These results indicate that the magnitude of any possible FU incorporation into any RNA fraction examined was less than about 4 % of the uracil present.

TABLE III

EFFECT OF 5-HALOGENOPYRIMIDINES ON THE FORMATION OF TMV IN EXCISED LEAF DISKS

Compound	*Concentration %*	*Virus yield-percent of control*
H_2O	—	100
5-Fluorouracil	0.1	50 ± 5
5-Fluorouridine	0.01	50 ± 5
5-Fluoro-2-thiouracil	0.1	52 ± 10
5-Fluoroorotic acid	0.05	41 ± 5
5-Fluoroorotic acid	0.025	52 ± 5
5-Fluoroorotic acid	0.01	58 ± 5
5-Fluoroorotic acid	0.001	88 ± 5
5-Chlorouracil	0.1	100 ± 5
5-Chlorouracil	0.2	100 ± 5
5-Bromouracil	0.1	93 ± 5
5-Iodouracil	0.1	90 ± 5

Inhibitory effects of other halogenated pyrimidine derivatives

The ribonucleoside of FU, 5-fluoro-1-β-D-ribofuranosyluracyl (fluorouridine), was found to be an inhibitor of the formation of TMV. The molar concentration of the nucleoside required for 50 % inhibition was about one-tenth of the equivalent concentration of the aglycone (Table III and Fig. 3). Fluorodeoxyuridine (5-fluoro-2'-deoxyuridine) was, comparatively, an ineffective inhibitor. These results are shown in Fig. 3. 5-fluoroorotic acid was also found to be an inhibitor of the formation of TMV under the conditions of the present assay (Table III).

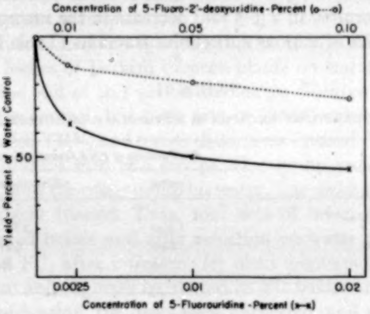

Fig. 3. Effects of 5-fluorouridine and 5-fluorodeoxyuridine on the formation of TMV in isolated disks of Turkish tobacco leaves.

The replacement of the oxygen in the 2-position of FU by sulfur did not decrease the inhibitory activity of the molecule. Incubation of leaves on a 0.1 % solution of 2-thio-5-fluorouracil also caused about a 50 % reduction in the yield of TMV (Table III).

The introduction of halogens other than fluorine into the 5-position of the uracil molecule resulted in virtually complete disappearance of inhibitory properties (Table III).

Incorporation of derivatives of FU into TMV-RNA

It was of interest to ascertain whether the inhibition of TMV growth by 5-fluoroorotic acid and 5-fluorouridine was accompanied by the incorporation of FU into the RNA of the virus. Accordingly, 114-mg and 89-mg lots of TMV were prepared from infected Turkish leaf disks incubated in the presence of 0.05 % 5-fluoroorotic

TABLE IV

INCORPORATION OF DERIVATIVES OF 5-FLOROURACIL INTO TMV–RNA

Compound[*]	*Concn. in incubation medium*	*Fluorouracil content of virus RNA*[**]
5-Fluoroorotic acid	0.05 %	43 %
5-Fluorouridine	0.01 %	19 %

[*] The concentrations of compounds used were found to result in an inhibition of about 50 % in the yield of virus.

[**] Expressed as percent of normal uracil content.

acid and 0.01 % fluorouridine solutions, respectively. Samples of RNA prepared from these lots of virus were hydrolyzed with alkali and examined for the presence of the 2′ and 3′ phosphate of 5-fluorouridine as previously described[5]. The results, as given in Table IV, indicate that each of these inhibitory compounds leads to the formation of TMV-RNA which contains 5-fluorouracil.

There was some indication of the formation of an abnormal TMV-RNA when infected leaves were incubated on a solution of 2-thio-5-fluorouracil; however, the results were complicated by the instability of the 2-thiosubstituent. No incorporation (less than 4 % of the uracil) was observed in the case of 5-chlorouracil[12].

Reversal of FU inhibition

Attempts were made to reverse the action of a 0.1 % solution of FU by the simultaneous addition of various compounds. It was found that uracil and thymidine at molar concentrations up to 10 times that of the FU had little or no effect on the formation of TMV. On the other hand, uridine had a definite effect on the production of virus, and at high concentrations of uridine the effect of FU was completely reversed as shown in Table V.

TABLE V

EFFECT OF URIDINE ON THE INHIBITION OF TMV FORMATION BY 5-FLUOROURACIL

Sample No.	*5-Fluorouracil concn. μ moles/ml*	*Uridine concn. μ moles/ml*	*Virus yield percent of control*	
			Expt. 1	*Expt. 2*
1	0	0	100	100
2	7.7	0	60 ± 5	53 ± 5
3	0	7.7	100 ± 5	101 ± 5
4	7.7	7.7	73 ± 5	74 ± 5
5	0	77	100 ± 5	103 ± 5
6	7.7	77	100 ± 5	101 ± 5
7	0	154	—	105 ± 5
8	7.7	154	—	101 ± 5

DISCUSSION

The synthesis of the uridylic acid moiety of tobacco leaf RNA and TMV-RNA is probably similar to the overall pathway which has been demonstrated in mammals and bacteria[13]. This scheme may be presented as follows:

$$\begin{tikzcd}
\text{Small precursors} \arrow[r] & \text{Orotic acid} \arrow[r] & \text{Orotidine-5'-phosphate} \arrow[d] & \\
\text{Uracil} \arrow[r] & \text{Uridine} \arrow[r] & \text{Uridylic acid} \arrow[r] & \text{RNA}
\end{tikzcd}$$

The levels of phosphorylation at which some of these reactions take place is unknown. The pathway from uracil is probably of minor importance except in the presence of exogenous uracil or uracil analogues. The 5-fluoro-analogues of orotic acid and uridine are closely related to the intermediates of the *de novo* synthesis of uridylic acid and should be readily converted into nucleotides of fluorouridine. It has been found that FU, 5-fluoroorotic acid, and 5-fluorouridine are inhibitors of TMV formation (Tables I and III) and lead to the formation of TMV-RNA containing FU (Tables I and IV). According to the above scheme it may be postulated that these compounds are metabolized to a common pool of fluorouridine nucleotides which are responsible for the similar behavior of these three compounds in the present system. It should be noted that synthetic nucleotides of FU have been prepared[14], so that it should be possible directly to observe their effects in cell free systems. The close relation of FU to the above scheme of *de novo* biosynthesis of uracil is also shown by the reversal of FU inhibition of TMV production by high concentrations of uridine (Table V). At the time these experiments were in progress the fluoro compounds labeled with radioactive isotopes were not available, so that the question of the relative permeability of the plant cells to these compounds could not be readily ascertained.

The incorporation of radiophosphorus into the subcellular components was also inhibited by FU (Table II). This finding indicates that the rates of turnover and synthesis of RNA in isolated leaf segments are decreased by the presence of a pool of fluorouracil nucleotides. In the case of chloroplast RNA some indication of the incorporation of a small amount of FU was obtained. It is not known whether this questionable and small incorporation of FU may be ascribed to a slower rate of synthesis and/or turnover of plant RNA as compared to viral RNA, or to the operation of more selective mechanisms.

Some insight into the mode of virus production was obtained with the use of various halogenated uracil analogues. Compounds such as 5-chloro, 5-bromo, and 5-iodouracil[15, 16], which are known to produce abnormal DNA molecules in *E. coli*, were virtually without effect on the formation of TMV in isolated disks of Turkish tobacco. The deoxyribonucleoside, 5-fluoro-2′-deoxyuridine, had only a slight inhibitory action under the conditions of the present assay in contrast to the striking activity of the corresponding ribonucleoside, 5-fluorouridine (Fig. 3). In mammalian[6] and bacterial[8] systems it has been found that 5-fluoro-2′-deoxyuridine-5′-phosphate is an extremely potent inhibitor of the thymidylate synthetase system. The action of FU or its derivatives in some of the latter systems may be partially[8], or completely[7], reversed by thymine or thymidine. In tobacco leaves infected with TMV, thymidine at 10 times the molar concentration of 0.1 % FU has no effect on the inhibitory action of the uracil analogue. Uridine, on the other hand, at high concentrations produces a complete reversal of the inhibition by FU (Table V). Although there is need for more direct evidence, the results obtained with the above series of compounds suggest that the formation of new molecules of deoxyribonucleic acid does not play an important role once the TMV infection is initiated in a tobacco leaf.

The results obtained with the 5-fluoropyrimidines in the present studies in some respects are similar to those obtained by HEIDELBERGER and colleagues[6] in an extensive series of investigations aimed at elucidating the mechanism of the inhibition of the growth of some neoplastic tissues by these compounds. These workers found that FU, 5-fluoroorotic acid, and 5-fluorouridine were converted into a pool of 5-fluorouridine nucleotides and, moreover, some conversion to 5-fluoro-2′-deoxyuridine monophosphate was observed. In a variety of tissues FU, 5-fluoroorotic acid, and 5-fluorouridine were incorporated into RNA (but not the DNA) as FU in amounts which approached a maximum of 6 % of the uracil content. Under certain conditions FU inhibited the incorporation of [^{32}P]phosphate into a variety of tissues *in vivo*. The results of the studies by the Wisconsin workers indicate that the mechanism of action of the 5-fluoropyrimidines in mammalian cells is complex and possibly involves the formation of acid soluble nucleotides, interference with the synthesis and function of RNA, and inhibition of DNA synthesis by blockage of the methylation of thymidine precursors.

The production of TMV in the isolated leaf system used in the present experiments was very sensitive to the time interval between infection and exposure to FU (Figs. 1 and 2), and the effect of FU decreases before the bulk of virus is synthesized. Since the yield of TMV in normal as well as in FU cultivated leaf disks reaches a maximum after 6-7 days, the inhibition of virus formation by FU seems to be absolute and not merely the reflection of a decrease in the rate of virus production. The experiments of Siegel and Wildman[17] indicate that on leaves of *Nicotiana glutinosa* a period of 5 h is required before a decrease of the u.v. sensitivity of the system indicates that the initial infecting TMV particle has given rise to a multiple number of infectious centers. In the case of systemic infection in Turkish tobacco, u.v. sensitive centers will probably be associated with the emergence of new loci of TMV formation during the spread of the infection. The presence of fluorouracil nucleotides during these crucial early stages would be expected to inhibit or completely abolish the formation of sites for virus synthesis. Once the mechanism for TMV formation is established in a site, newly introduced fluorouracil ribonucleotides would merely be polymerized into molecules of TMV-RNA.

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THYROIDAL IODIDE TRANSPORT

I. CARDIAC GLYCOSIDES AND THE ROLE OF POTASSIUM

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SUMMARY

Iodide concentrating ability of sheep thyroid slices, measured at approximately 80–90 % equilibrium between intra- and extracellular iodide is depressed by various cardiac glycosides or their aglycones at concentrations of 10^{-7} *M* to 10^{-6} *M*. Quinidine is active at 10^{-4} *M* to 10^{-3} *M*. Beef, guinea pig and human thyroid glands are sensitive in the same concentration range, whereas certain other tissues are very much less sensitive.

Increased extra-cellular concentrations of potassium or rubidium ions can overcome the inhibition produced by ouabain in sheep thyroid slices, and K^+ can similarly reverse the depression in iodide concentrating ability resulting from the leaching of sheep thyroid slices in K^+-free Ringer solution. It is suggested that potassium is required for the establishment and maintenance of a concentration gradient for iodide in thyroid tissue.

INTRODUCTION

One of the important properties of thyroid tissue is its ability to concentrate iodide ion from the surrounding medium against a concentration gradient. Depending on the functional status of the tissue and on the species employed, tissue to medium iodide ratios may be as high as 300 or more. It has been assumed that this gradient is the result of "active" transport of iodide into the cell. The following findings, both *in vivo* and *in vitro*, taken together, may be considered as evidence supporting such an assumption: (a) despite intensive search, no bound form of iodide has been detected which would remove it from the equilibrium with extracellular iodide[1,2], (b) phosphate-bond energy seems to be required, as judged by the inhibition of iodide concentration produced by anaerobiosis and by agents that uncouple oxidative phosphorylation[3,4], (c) the concentrating mechanism exhibits saturation at extracellular iodide concentrations of approximately 10^{-3} *M*[2,5], (d) anions such as SCN^- or ClO_4^- can compete with iodide for the concentrating mechanism[6,7], (e) the thyroidal concentration gradient for iodide is abolished at low temperatures[3] (unpublished observations). Aside from the above factors, little information is available on the mechanism of iodide concentration in thyroid tissue.

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It has recently been possible, by the use of K^+-depleted erythrocytes, to show that K^+ influx can be inhibited by low concentrations of the cardiac glycosides[8–10]. This effect appears[10,11] to be the result of competition between glycoside and K^+. The transport of Cl^- ion in the colon is also inhibited by cardiac glycosides[12]. As a result of such observations we tested the effect of these agents on thyroidal iodide concentration and found recently[13] that glycosides such as digitoxin and strophanthin, or the aglycone strophanthidin, are able to inhibit this process. In the present paper these findings are extended and an attempt is made to show that the depression of thyroidal iodide concentration by the cardiac glycosides is linked to an interference with K^+ movement. The presence of an "adequate" level of K^+ ion is probably required for "active" entry of iodide into the thyroid cell.

MATERIALS AND METHODS

Sheep thyroid glands were obtained at the local abattoir and we should like to thank F. Ensten & Co., Totteridge, London, for their generous co-operation. The glands were kept on ice, trimmed free of extraneous tissue and sliced free-hand. Whenever possible, slices from the same animal were used. In large experiments this was not possible and slices were pooled. Under these conditions the inherent error is about 10 % and for this reason all points were determined in triplicate unless otherwise noted. Human thyroid tissue, obtained at surgery, was made available through the kindness of Drs. D. DONIACH and I. ROITT of the Middlesex Hospital, London. Rat and guinea pig thyroids were obtained from male adults pre-treated for 8–10 days with thiouracil, or for two days with one I.U. subcut. daily of thyrotropin in order to enhance the iodide concentrating efficiency of the small amounts of tissue available. Experiments on salivary tissue were carried out on slices of submaxillary glands obtained from male mice of the C3H or Parkes strains weighing 35–45 g. Conditions were otherwise those used for thyroid tissue. Growing plants of *Fucus vesiculosus* were collected on the chalk cliffs near Rottingdean, Sussex, brought to the laboratory and incubated in sea water containing 10^{-3} *M* methyl mercaptoimidazole but no added iodide at 20° according to KLEMPERER[14]. Otherwise conditions were the same as for animal tissues.

Slices approximately 0.3 mm thick were rinsed for 5 min in iodide-free Ringer solution and were then incubated in a Ringer-phosphate medium pH 7.4 containing: 0.148 *M* Na^+, $4 \cdot 10^{-3}$ *M* K^+, $1 \cdot 10^{-3}$ *M* Ca^{++}, $1.33 \cdot 10^{-3}$ *M* Mg^{++}, $1.33 \cdot 10^{-3}$ *M* $SO_4^=$, $8.6 \cdot 10^{-3}$ *M* phosphate, 0.154 *M* Cl^-, and $1 \cdot 10^{-6}$ *M* I^- (added). Carrier-free ^{131}I iodide was added at approx. 1–2 μC/100 ml. In order to block the further utilization of iodide for hormone biosynthesis, 1-methyl-2-mercaptoimidazole was added to a final concentration of $1 \cdot 10^{-3}$ *M*. Under these conditions $> 99.4\%$ of thyroidal radioiodine was iodide as judged by trichloracetic acid solubility, and no organic iodine was found by chromatography. Slices were incubated at 37° with air as the gas phase and shaken at a rate of about 90/min. Incubation was carried out for 75–90 min at which time about 80 % to 90 % of the equilibrium value had been attained (Fig. 1). With very active glands equilibration either did not occur even in 4 h or occurred very late, and experiments with slices of such glands were not used.

Except where noted, 100–150 mg of tissue (wet wt.) were used per 3 ml of medium. After incubation, the slices were blotted and re-weighed. As weight is lost during the experiment, all results are expressed in terms of the final wet weight and are listed as the ratio of counts of ^{131}I/g of tissue to the counts/ml of medium—hereafter referred to as the T/M [I⁻], whose values generally ranged from 20–40. At medium iodide concentrations of $10^{-6}\,M$ the iodide content of pre-rinsed slices constitutes only a small fraction of the total iodide pool, hence exchange may be neglected and the T/M [I⁻], as measured by ^{131}I, represents a meaningful concentration gradient.

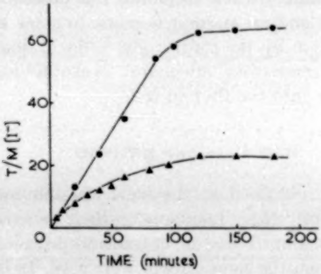

Fig. 1. The time course of iodide equilibration in sheep thyroid slices. T/M [I⁻] represents the counts/min/g of slices: counts/min/ml medium. ● & ▲ are thyroids from different sheep.

When control values were less than 10–12, the experiment was discarded (except in the case of mouse submaxillary glands). Results in Tables I and II are expressed as the concentration of drug required to obtain a 50 % depression in the T/M [I⁻] as determined from log-dose response of the type shown previously[13].

Ouabain, digitoxin, digitoxigenin, strophanthin K, strophanthidin and α-angelica-lactone were obtained commercially. Lanatoside B was generously supplied by Dr. R. A. Ellis of Sandoz Products Ltd., London, and dihydrodigoxin through the kindness of Dr. G. H. Acheson, University of Cincinnati. Except for ouabain, the glycosides were dissolved in ethanol diluted with water and added to the flasks in 0.1 of the final volume to yield a final ethanol concentration of 1–2 %. Control flasks contained equivalent quantities of ethanol. Because of its greater solubility in water ouabain was used in most of the studies. The molarity of ouabain was corrected for 20 % water of crystallisation. The other compounds were assumed to contain no water.

The veratrine alkaloids: veratridine, veratramine, cevadine and protoveratrine were generously supplied by Dr. O. Krayer, Harvard University. They were dissolved in ethanol and HCl and neutralized to pH 7.4 with 0.1 M Tris buffer. Control flasks received equivalent additions. Desoxycorticosterone was the reference standard, eserine sulfate of B.P.C. grade and RbCl and CsCl of Lab. Reagent Grade. De-ionized water with a resistance of approx. 3 megohms/cm was used throughout.

RESULTS AND DISCUSSION

Several different cardiac glycosides are able to reduce the equilibrium T/M [I⁻] at concentrations of 10^{-7} to $10^{-6}\,M$. As can be seen from Table I, in which glycoside effects on iodide concentrating ability as determined in this study are compared with values from the literature on these effects on K⁺ flux, these concentrations are

TABLE I

COMPARISON OF THE INHIBITORY LEVELS OF CARDIAC GLYCOSIDES ON THYROIDAL IODIDE CONCENTRATION AND ON POTASSIUM TRANSPORT IN ERYTHROCYTES

	*I_{50} of T/M [I⁻]** sheep thyroid slices*	*I_{50} of K^+ transport** human red cells from* KAHN[15]	*$K_{G\text{-}1}$ of K^+ transport*** human red cells from* SOLOMON *et al.*[10]
	M	*M*	*M*
Strophanthin K	$2\text{–}5 \cdot 10^{-7}$		
Strophanthidin	$2 \cdot 10^{-7}$	$1.2 \cdot 10^{-7}$	$9.9 \cdot 10^{-8}$
Digitoxin	$2 \cdot 10^{-7}$	$1.6 \cdot 10^{-6}$	
Digitoxigenin	$1\text{–}2 \cdot 10^{-6}$	$1.6 \cdot 10^{-6}$	$2.4 \cdot 10^{-7}$
Ouabain	$3 \cdot 10^{-7}$	$3\text{–}5 \cdot 10^{-8}$	$1.7 \cdot 10^{-8}$
Lanatoside B	$7 \cdot 10^{-7}$		$1.2 \cdot 10^{-6}$
Dihydrodigoxin	$3\text{–}4 \cdot 10^{-5}$	$9 \cdot 10^{-5}$	
α-Angelicalactone	$7\text{–}8 \cdot 10^{-4}$	$2 \cdot 10^{-2}$	

* Concentration required to reduce the 80–90% equilibrium concentration gradient of $^{131}I^-$ (10^{-8} *M*) to half of the control value at $4 \cdot 10^{-3}$ *M* K^+.

** Concentration inhibiting K^+ loss from plasma into cold stored human erythrocytes to half of the control value.

*** K_{G-1} is the dissociation constant of glycoside from its binding site on the human erythrocyte and is analogous to the K_i used in enzyme inhibition.

TABLE II

THE EFFECT OF OUABAIN ON VARIOUS IODIDE CONCENTRATING TISSUES

Tissue	*I_{50} of T/M [I⁻]**
Thyroid	*M*
Sheep	$3 \cdot 10^{-7}$
Beef	$2 \cdot 10^{-7}$
Guinea Pig**	$2 \cdot 10^{-6}$
Rat**	$2\text{–}3 \cdot 10^{-4}$
Human (Thyrotoxicosis)***	$9 \cdot 10^{-8}$
Human (Recurrent thyrotoxicosis)***	$1\text{–}2 \cdot 10^{-7}$
Other	
Submaxillary (Mouse)	$8 \cdot 10^{-4}$
Fronds of *Fucus vesiculosus*	$\gg 3.5 \cdot 10^{-3}$

* Concentration of ouabain required to reduce the T/M [I⁻] to one half of the control value.

** Curves on duplicate points. 10–15 mg of thyroid tissue/3 ml of Ringer solution.

*** Single experiment, points determined in triplicate.

comparable to those required to reduce K^+ influx to K^+-depleted erythrocytes*, but a more detailed inspection reveals that the spread in relative potencies is not nearly so great in the case of the thyroid cell as it is with the red cell. The aglycones, strophanthidin and digitoxigenin, are also effective, although the latter is less potent by an order of magnitude.

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* The values of KAHN[15] were calculated on the assumption that no change in K^+ concentration represents 100% inhibition. If they had been corrected for reverse flux they would have to be increased by a factor of 1.7. Thus the 50% inhibition point for ouabain becomes $6.8 \cdot 10^{-8}$ *M*. When the dissociation constants of SOLOMON *et al.*[10] are converted to 50% inhibitory concentrations, these values will rise from a K_{G-1} of $1.7 \cdot 10^{-8}$ to a 50% point of $5.6 \cdot 19^{-8}$ *M*. ^{131}I was measured as the equilibrium T/M [I⁻] in the present study, hence such corrections may not be directly applicable. Since it has not been possible to reduce the T/M [I⁻] to less than 1–2, such corrections would lower the values for 50% inhibition by approx. 20%.

Unsaturation in the α-angelicalactone ring appears to be of importance since dihydrodigoxin, in which the α-β double bond is reduced to yield the γ-butyrolactone derivative, is less potent by two orders of magnitude. Glynn[11] has also shown that reduction of the lactone (a 2-pyridone derivative) of *Scillaren A* leads to considerable diminution in inhibitory activity on K^+ transport. Other deviations in the response of the T/M [I^-] are seen in the inhibitory effect of the steroid-free lactone, α-angelicalactone which is 25 times more active on the thyroid gland than in erythrocytes. However, the higher concentrations of this material tended to bleach the thyroid slices and the effect may well be of a different nature from that of the glycosides.

Since it has been postulated that quinidine acts by way of an interference in K^+ movement (K^+ efflux from the natrium[16] and K^+ influx in cold-stored erythrocytes[17]), it was of interest to test it in the present system. The concentration required for a 50 % depression of the T/M [I^-] was $3 \cdot 10^{-4}$ *M*, an inhibitory potency of the same order of magnitude in the above systems.

Of the other compounds investigated some of the veratrine alkaloids were slightly active at the single high concentration tested ($2.6 \cdot 10^{-4}$ *M*): veratramine 28 %, veratridine —9 %, cevadine 10 % and protroveratrine 17 % of the control values. These findings are similar to those of Kahn and Acheson[8] who found these compounds to be inactive on cation transport in erythrocytes at somewhat lower concentrations ($1.5 \cdot 10^{-5}$ *M* to $2 \cdot 10^{-4}$ *M*). $2 \cdot 10^{-4}$ *M* deoxycorticosterone was inactive in agreement with the results of Glynn on red cell K^+ transport[11] and $6 \cdot 10^{-4}$ *M* eserine led to a slight increase in the T/M [I^-].

A comparison was made of thyroids of various species as well as of certain other tissues known to concentrate iodide. As can be seen in Table II, thyroid from a number of species other than the sheep possess sensitivities to ouabain in the same range of concentration. The most sensitive gland was that from a 35-year old woman with thyrotoxicosis. Surprisingly, rat thyroid tissue was quite resistant to ouabain, a finding which raises doubts about the difference in the concentrating mechanism of a rat thyroid tumor recently postulated[4] from that of normal tissue because comparison was made with sheep tissue.

Since mouse salivary tissues can concentrate iodide[18] the marked resistance of mouse submaxillary slices to ouabain was unexpected. It was impossible to obtain slice medium ratios greater than 4 in mice of the C3H or Parkes strains, but it is doubtful that this can explain the lack of effect. Iodide concentrating ability in the brown alga, *Fucus vesiculosus*, was not inhibited by high concentrations of ouabain despite T/M [$^-$] value of 100 or greater. Because of the high K^+ levels (see below) in sea water, experiments were carried out with sea water diluted 1:2 with K^+-free Ringers solution—again ouabain was without effect. Whether or not the findings on non-thyroid iodide concentrating tissues imply a different concentrating mechanism remains a moot point. Preliminary experiments with ^{42}K indicate that ouabain produced roughly similar degrees of inhibition of entry of ^{42}K or ^{131}I into the various tissues tested. Thus, although net K^+ fluxes were much greater than iodide fluxes since the external K^+ concentration was about 4000 times that of the I^- concentration, 50 % inhibition for ouabain occurred at about $3 \cdot 10^{-7}$ *M* for both ^{42}K and ^{131}I, and between 10^{-3} *M* and 10^{-4} *M* in mouse submaxillary slices for both isotopes.

^{42}K entry into *Fucus vesiculosus* was not influenced by $3.3 \cdot 10^{-3}$ *M* ouabain, corresponding to the lack of effect on ^{131}I transport. The results of Table II therefore suggest variations in tissue sensitivity to ouabain and conclusions about alternative iodide concentrating mechanisms are not warranted.

The relation of the iodide concentrating ability to K^+ transport

The above results emphasize the marked similarity between glycoside inhibition of erythrocyte K^+ transport and of thyroidal iodide concentration. Since GLYNN[11] and SOLOMON *et al.*[10] had shown that the glycosides probably act by competitive inhibition with K^+ on the transport site, attempts were made to see if K^+ ions could overcome the depression in the T/M [I^-] produced by ouabain. Accordingly, sheep thyroid slices were incubated with progressive increments of K^+. As can be seen from Fig. 2, K^+ readily restored the T/M [I^-] that had been depressed by ouabain. In view of the fact that K^+ increased iodide concentration to some extent in thyroid slices not treated with ouabain (Table IV) the corrected slope would be somewhat lower.

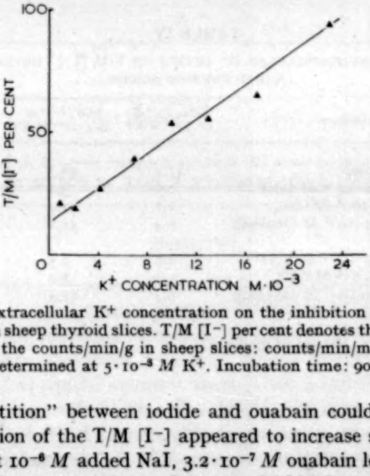

Fig. 2. The effect of extracellular K^+ concentration on the inhibition by $6.4 \cdot 10^{-7}$ *M* ouabain of iodide concentration in sheep thyroid slices. T/M [I^-] per cent denotes the proportion of the control value (no ouabain) of the counts/min/g in sheep slices: counts/min/ml in the medium. Controls determined at $5 \cdot 10^{-3}$ *M* K^+. Incubation time: 90 min.

Direct "competition" between iodide and ouabain could not be demonstrated, in fact, the depression of the T/M [I^-] appeared to increase slightly with increasing iodide load. Thus at 10^{-6} *M* added NaI, $3.2 \cdot 10^{-7}$ *M* ouabain led to a 53 % depression below the ouabain-free preparation and at $2 \cdot 10^{-5}$ *M* NaI to a 66 % depression in the T/M [I^-]. Attempts were made to determine whether or not other cations could likewise overcome ouabain inhibition in view of the known ability of Rb^+ and Cs^+ to replace K^+ in certain systems. It can be seen (Table III) that Rb^+ can replace K^+ in overcoming ouabain inhibition, that Cs^+ has a slight or questionable effect, but that none of the other cations added to the Ringer solution have any effect. This recalls the ability of these ions to replace K^+ in certain other systems where membrane phenomena are involved [19,20] and differs from the replacement of K^+ in enzyme systems[21–23] where the NH_4^+ ion usually has high activating activity.

While these findings suggest a competition between ouabain and K^+ for the depression of the T/M [I^-], it must be remembered that K^+ also enters the cell by several processes, (*e.g.* diffusion), which are not sensitive to the cardiac glycosides[10–11].

TABLE III

EFFECT OF MONOVALENT CATIONS ON THE INHIBITORY ACTION OF OUABAIN ON THYROIDAL IODIDE ACCUMULATION

Sheep thyroid slices incubated in Ringer solution containing $1 \cdot 10^{-6}$ *M* NaI and $8 \cdot 10^{-7}$ *M* ouabain. All salts were added as the chlorides, to a concentration of 0.02 *M* of the added salt.

Additional cation	*Per cent of control T/M [I⁻]**
None (Control – no ouabain)	100
None	28
Li^+	27
NH_4^+	26
Na^+	37
K^+	96
Rb^+	90
Cs^+	41

* Ratio of ^{131}I in 1 g of thyroid tissue to the ^{131}I in 1 ml of medium.

TABLE IV

THE EFFECT OF HIGH EXTRACELLULAR K^+ LEVELS ON T/M $[I^-]^*$ DEPRESSION IN SHEEP THYROID SLICES

Additions	*T/M [I⁻]* at $3.6 \cdot 10^{-3}$ M K+*	*T/M [I⁻]* at $19.4 \cdot 10^{-3}$ M K+*
None	41.4	48.3
$3.1 \cdot 10^{-3}$ *M* DNP**	2.6	3.1
$3 \cdot 10^{-4}$ *M* ClO_4^-	1.1	1.1
$6.4 \cdot 10^{-7}$ *M* Ouabain	6.4	55.2
$8.3 \cdot 10^{-5}$ *M* DNP**	2.0	2.3
$1 \cdot 10^{-4}$ *M* ClO_4^-	1.2	1.2
$6.4 \cdot 10^{-7}$ *M* Ouabain	6.4	27.4

* ^{131}I/g of thyroid tissue: ^{131}I/ml of medium.
** 2,4-dinitrophenol.

The possibility thus remained that high extracellular K^+ concentrations could lead to enough K^+ diffusion to permit apparently normal I^- transport. If this were the case, then it should be possible to overcome T/M $[I^-]$ depression produced by other agents such as DNP but not that produced by agents that compete "directly" with iodide *e.g.* SCN^- or ClO_4^-. High extracellular K^+ levels were able to increase the DNP depressed T/M $[I^-]$ only slightly and did not influence the ClO_4^- effect (Table IV). In contrast, ouabain inhibition of the T/M $[I^-]$ was reversed by K^+. It is not likely therefore that simple diffusion contributes a large fraction to the K^+ reversal of ouabain inhibition of the T/M $[I^-]$.

It might be argued that the effect of the ouabain is merely one of depressed rates and that the T/M $[I^-]$ would approach the control value upon prolonged incubation. However, the depression in the T/M $[I^-]$ was identical at 75 and 180 min, suggesting that ouabain in some way alters the equilibrium between intra- and extra-cellular iodide.

Attempts to study the T/M $[I^-]$ in the absence of K^+ were successful in only about half the cases. Incubation in K^+-free Ringer solution caused only a slight lowering in iodide-concentrating ability. It seemed possible that under these conditions enough K^+ leaked out of the thyroid slices to supply the "required" amount of K^+. Slices were therefore leached for four successive 15 min-periods in K^+-free Ringer solution at 20° and were then incubated at 37° in K^+-free Ringer solution to which varying amounts of K^+ had been added. It can be seen from Table V that this procedure reduced the T/M [I^-] to about 60 % of the control value. While other materials besides K^+ were, no doubt, also leached out, increasing levels of added K^+ restored the T/M [I^-] to the control value. It is not known why this procedure failed to demonstrate a K^+-dependence in half the experiments; it may possibly due be to failure to wash out sufficient intra-cellular K^+.

TABLE V

THE EFFECT OF LEACHING AND K^+ REPLACEMENT ON IODIDE CONCENTRATION IN SHEEP THYROID SLICES

*K^+ replacement**	*T/M [I^-]*
—	12.2
$1 \cdot 10^{-5}$ *M* K^+	15.2
$2.5 \cdot 10^{-4}$ *M* K^+	17.4
$5 \cdot 10^{-3}$ *M* K^+	21.6
$5 \cdot 10^{-3}$ *M* Control (Not leached)	19.0

* Slices were leached in K^+-free Ringer's solution for four 15-min periods at 20° and were then incubated at 37° for 90 min in Ringer's solution to which varying concentrations of K^+ had been added.

COMMENT

In the present study it has been found that K^+ ions are involved in the establishment and maintenance of a concentration gradient for iodide in thyroid tissue. This conclusion is based on the following evidence: (a) Known inhibitors of K^+ transport in other tissues (cardiac glycosides, quinidine) depress thyroidal iodide concentrating ability; the T/M [I^-], at similar concentrations (Table I and II), (b) ouabain inhibits ^{131}I and $^{42}K^+$ entry into various tissues to roughly the same extent despite widely different sensitivities of these tissues to the inhibitor, (c) ouabain depression of the T/M [I^-] can be overcome by increasing the K^+ concentration of the medium but not by increasing the I^- concentration (Fig. 1, Tables III and IV). (d) The depression in the T/M [I^-] produced by leaching sheep thyroid slices in K^+-free Ringer solution is reversed by K^+ replacement (Table V). The latter two findings would, moreover, tend to rule out a similar glycoside effect on two otherwise independent processes.

If we assume that K^+ is intimately involved with iodide transport and the maintenance of the T/M [I^-] a number of questions arise concerning its role in this process: (a) K^+ transport appears to be linked to an opposing Na^+ transport in a number of systems and low concentrations of extra-cellular K^+ may be required for Na^+ efflux[11, 24, 25]. It is clear therefore that in the present system, Na^+ efflux must be considered as a possible factor in the transport of iodide. It is also possible that the intra-cellular level, *per se*, of these ions is important. (b) High intra-cellular and low extracellular K^+ concentrations could establish a potential difference which would lead to the transport of I^-. In the case of Cl^- transport across frog skin, Ussing[26] has shown that the potential difference established by Na^+ is adequate to explain the observed movement of Cl^- ion. The mere operation of an electro-chemical potential cannot, however, be the only driving force for iodide transport since the smaller Br^- ion (Crystal radii: $Br^- = 1.95$ Å, $I^- = 2.16$ Å[28]; and the partial ionic molal volumes in dilute aqueous solution are: $Br^- = 25.1$ cc/mole and $I^- = 36.7$ cc/mole[29]). shows only very slight concentration in thyroid tissue[27]. An additional objection is that saturation of the iodide concentrating mechanism would not be expected at the relatively low absolute concentrations of iodide usually found. (d) The T/M $[I^-]$ has been used here as a convenient screening method for the inhibitory potencies of the cardiac glycosides. As it represents 80–90 % of the equilibrium state, conclusions about iodide flux are complicated by the non-linear iodide accumulation and can hence be compared only indirectly to the K^+ fluxes in erythrocytes. It is hoped that studies now in progress on the kinetics of the system, and on the stoichiometry between K^+ and I^- will allow direct comparison and shed light on the above problems.

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SUR LE MÉTABOLISME DU SULFOCONJUGUÉ DE LA 3,5,3'-TRIIODOTHYRONINE CHEZ LE RAT

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SUMMARY

Metabolism of the sulfoconjugate of 3,5,3'-triiodothyronine in rat

1. The comparative metabolism of 3,5,3'-triiodothyronine (T_3) and of its sulfoconjugate (ST_3) have been studied in the thyroidectomized Rat. The former is degraded more rapidly than the latter, which the cells concentrate much less intensely.

2. The different aspects of ST_3 metabolism, among others the formation in liver of the glycuroconjugate of T_3 from it, the excretion of iodide after its injection, the fact that it exists in plasma but not in urine, all lead to the supposition that it is partially hydrolyzed by some arylsulfatases in tissues and that its hormone moiety is deiodinated.

3. ST_3 probably plays the part of a storage form of T_3 which the cells can use after hydrolysis of the ester bond.

INTRODUCTION

L'ester sulfurique (ST_3) de la 3,5,3'-triiodothyronine, prenant naissance dans le foie après administration au Rat thyroïdectomisé de 3,5,3'-triiodo-L-thyronine (T_3) à dose physiologique, est excrété par la bile et présent dans le plasma[1]. Nous nous sommes proposé d'étudier son métabolisme, afin de préciser la signification physiologique de la sulfoconjugaison de T_3, en la comparant à celle, aujourd'hui bien définie, de la glycuroconjugaison de la même hormone[2]. Dans ce but, nous avons étudié comparativement le métabolisme de ST_3 et de T_3 administrés par voie veineuse ou duodénale, le transport plasmatique, la répartition tissulaire et le transit hépatique de ces deux corps.

PARTIE EXPÉRIMENTALE

Techniques

Les recherches ont été poursuivies sur des rats ♂ (140–150 g) thyroïdectomisés depuis 20 jours au moins et maintenus à 24° C. ST_3 et T_3 marqués en position 3' par ^{131}I ont été préparés selon des techniques décrites antérieurement[1]; ils renfermaient moins de 1 % ^{131}I total sous forme minérale (^{131}INa) et ont été administrés en solution aqueuse (pH = 9.0). La radioactivité spécifique des produits utilisés était de 0.4–0.7 $\mu C/\mu g$ (mesures faites au compteur à scintillation, à cristal creux: NaI.Tl Tracerlab).

La séparation et l'identification des constituants iodés radioactifs de l'urine, du plasma et de la bile ont été opérées par électrophorèse sur papier et chromatographie, suivies de l'autographie des documents obtenus. L'électrophorèse a été opérée sur papier Whatman No. 1, en présence de solutions tampons appropriées (véronal sodique 0.2 M, de pH = 8.0; $CO_3(NH_4)_2$ 0.2 M, pH = 9.0) sous 200 V et 25 mA pendant 2 h dans le cas de la séparation des iodures, sous 120 V et 25 mA pendant 15 h pour celle de T_3 et ST_3 ajoutés au plasma. Dans ces conditions, la séparation des iodures d'une part et celle des fractions protéiques du sérum transportant ST_3 et T_3 ont été réalisées d'une manière satisfaisante. Deux solvants chromatographiques: le mélange collidine–eau (100 : 35) en atmosphère saturée d'NH_4OH (phase ascendante), et le n-butanol saturé d'NH_4OH 2 N (phase descendante), ont été employés sur papier Whatman No. 1. Le premier est particulièrement efficace pour séparer les deux produits, sulfurique et glycuronique, de la conjugaison de T_3 et le second pour individualiser T_3 en présence de ceux-ci.

Résultats expérimentaux

Excrétion urinaire et fécale d'^{131}I après administration de ST_3 et de T_3 par voie sanguine: Des rats répartis en deux lots de six ont reçu, les uns ST_3 marqué (0.6 µg/100 g de poids corporel), les autres T_3 (0.5 µg/100 g). Les urines et les fèces, recueillies dans des cages à métabolisme, ont été séparées toutes les 24 h pendant quatre jours et l'on a calculé la proportion des iodures marqués par rapport à ^{131}I urinaire total, après dosage radioélectrophorétique de ces sels. Dans tous les cas, plus de 70–90 % de la radioactivité de l'urine s'est montrée fixée aux iodures. Les résultats de ces essais ont été reportés sur la Fig. 1.

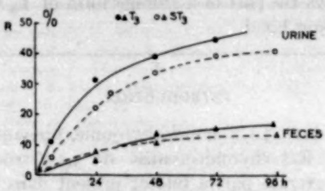

Fig. 1. Cinétique de l'excrétion urinaire et fécale d'^{131}I, chez le Rat thyroïdectomisé après injection par voie veineuse de [3'-^{131}I]T_3 et [3'-^{131}I]ST_3 à la dose de 0.5 et 0.6 µg/100 g de poids. Abscisses: temps en heures après l'injection; ordonnées: radioactivité de l'urine ou des fèces en % d'^{131}I administré.

L'excrétion urinaire d'^{131}I est très voisine dans les deux cas, quoiqu'un peu plus faible dans celui de ST_3; elle traduit la désiodation progressive de T_3 et de son ester. En aucun cas, ST_3 injecté n'a été retrouvé en quantité appréciable dans l'urine. Le passage d'une fraction de ce corps ou de dérivés organiques de celui-ci dans les matières fécales est relativement faible. Les fèces ne renferment pratiquement que des combinaisons iodées organiques et l'urine presque que des iodures. En effet, l'addition de sérum à l'urine ou à des suspensions aqueuses de fèces permet uniquement dans le dernier cas de précipiter ^{131}I avec les protéines par addition d'acide trichloracétique. La précipitation trichloracétique d'^{131}I avec des protéines sériques ajoutées à l'urine porte sur environ 5 % d'^{131}I total, les iodures demeurant alors en solution.

Excrétion biliaire de ST_3 et de T_3 administrés par voie sanguine ou duodénale: Cinq rats anesthésiés au pentothal et porteurs d'une fistule biliaire (tube de polythène) ont reçu en injection intraveineuse ST_3 (0.6 μg/100 g) ou T_3 marqués et l'on a recueilli par portions successives la bile s'écoulant de leur fistule. Les résultats obtenus, rassemblés dans la Fig. 2, montrent que l'excrétion de produits radioactifs est nettement moins intense et moins rapide après l'administration de ST_3 qu'après celle de T_3. La radioactivité des iodures biliaires, déterminée sur les autoradioélectrophorégrammes, demeure alors voisine de 5 % d'^{131}I total à la sixième heure et n'atteint que 9 % à la vingtième; aussi, y avait-il lieu d'étudier la nature des corps organiques iodés présents dans la bile des animaux traités par ST_3. Pareille étude avait déjà permis d'identifier, après administration de T_3[1,3], cette hormone elle-même, l'acide 3,5,3'-triiodothyropyruvique, le glycuroconjugué de T_3, ST_3 et de très petites quantités d'acide 3,5,3'-triiodothyroacétique, en dehors des iodures. La chromatographie d'échantillons de bile recueillis à des temps successifs a été opérée en collidine aqueuse (100 : 35) et en atmosphère saturée d'NH_4OH ou en *n*-butanol saturé d'NH_4OH 2 *N*. Un exemple des documents obtenus est reproduit sur la Fig. 3.

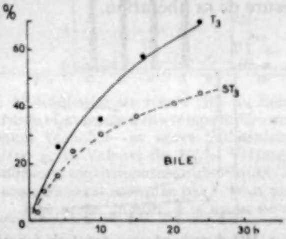

Fig. 2. Cinétique de l'excrétion billiaire d'^{131}I, chez le Rat thyroïdectomisé après injection intraveineuse de [3'-^{131}I]ST_3 (0.6 μg/100 g). Abscisses: temps en heures après l'injection; ordonnées: radioactivité de la bile en % d'^{131}I injecté.

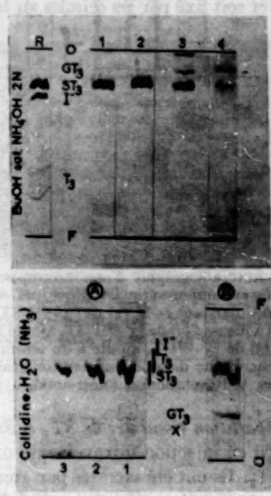

Fig. 3. Autoradiochromatogrammes de divers prélèvements de bile après injection intraveineuse de [3'-^{131}I]ST_3, analysés en *n*-butanol saturé par NH_4OH 2 *N* (I) et en collidine–eau (100 : 35) en atmosphère NH_3 (II, A). (1) bile de 0–1 h; (2) bile de 1–3 h; (3) bile de 3–5 h; (4) bile de 10–20 h. II, B: Autoradiochromatogramme de la bile des deux premières heures, après injection intraduodénale de 4 μg [3'-^{131}I]ST_3, analysée en collidine–eau en atmosphère NH_3. R = chromatogramme des corps de référence (T_3, ST_3 et I^-) en solution dans la bile de Rat non traité, et analysée en butanol ammoniacal.

Pendant les trois premières heures d'expérience, la bile ne renferme que ST_3 comme corps radioactif; on y trouve par la suite des iodures et le glycuroconjugué de l'hormone, GT_3. Le taux de GT_3 augmente progressivement jusqu'à renfermer près de la moitié d'^{131}I total vers la vingtième heure. ST_3 est donc moins intensément dégradé que T_3 par le foie et partiellement hydrolysé, car la formation hépatique de GT_3 ne peut avoir lieu qu'à partir de T_3 libre. La formation de GT_3 paraît plus rapide après injection intrapéritonéale de ST_3 (Fig. 3, IIB), ce qui peut tenir à une certaine hydrolyse de ce corps par l'arylsulfatase des bactéries intestinales.

Liaison aux protéines plasmatiques et métabolites sanguins de ST_3: Le transport plasmatique des hormones thyroïdiennes s'opère par combinaison de celles-ci à une protéine (TBP) migrant entre les α_1- et α_2-globulines lors de l'électrophorèse de celles-ci sur papier et à un groupe d'albumines, chez la plupart des Mammifères[4]. Nous avons recherché s'il en est de même de ST_3 ajouté à du sérum de Rat. On trouvera sur la Fig. 4 les autogrammes traduisant la migration électrophorétique de ST_3 et de T_3 en solution aqueuse et ajoutés au sérum, dans des conditions qu'indique la légende.

L'examen de ce document montre que ST_3 n'est pas transporté par la même globuline que T_3 (TBP) mais par des albumines. De plus, à quantités égales, T_3 est entièrement combiné à TBP, alors que ST_3 demeure en partie libre (présence d'une tache radioactive de ST_3 au-dessus de celle localisée sur les albumines, en 2, occupant la même position que celle de ST_3 en solution, en 1 (Fig. 4).

Quant aux constituants iodés radioactifs du plasma, 5 min, 3 et 6 h après l'injection de ST_3, leur analyse chromatographique n'a permis de déceler parmi eux que ST_3 et des iodures. T_3 n'a jamais été mis en évidence; mais il est possible qu'il se forme et soit fixé par les cellules au fur et à mesure de sa libération.

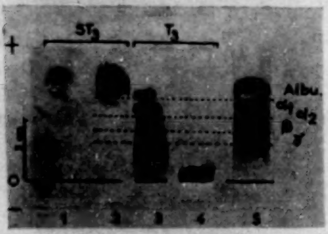

Fig. 4. Autoradiographie d'une électrophorèse sur papier en tampon véronal sodique, pH = 8,6 sous 120 V pendant 15 h; (1) de [3'-^{131}I]ST_3 en solution aqueuse; (2) de [3'-^{131}I]ST_3 ajouté à du sérum; (3) de [3'-^{131}I]T_3 ajouté à du sérum; (4) de [3'-^{131}I]T_3 en solution aqueuse. (5) Séparation électrophorétique dans les mêmes conditions que A, des fractions protéiques du sérum de Rat utilisé en A. Révélation au "noir-amide". O = origine; Alb. = albumines; α, β, γ = globulines.

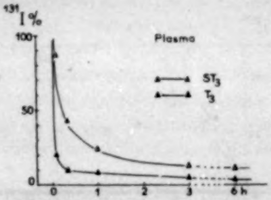

Fig. 5. ^{131}I dans le plasma total en fonction du temps et exprimé en % de la radioactivité injectée, après injection intraveineuse de [3'-^{131}I]T_3 et après celle de son ester sulfurique marqué de même (0.6 μg/100 g).

Répartition tissulaire de ST_3 et de T_3 administrés par voie sanguine· Quinze rats ont reçu en injection intraveineuse 0.6 μg ST_3/100 g de poids corporel et douze rats 0.6 μg T_3. Ils ont été sacrifiés par groupes de deux ou de trois, 5, 20, 60 et 180 min après le temps zéro et la radioactivité de leur sang et des divers tissus a été mesurée. Le rapport $R_I = \frac{^{131}\text{I/mg tissu}}{^{131}\text{I/mg plasma}}$ (espace ST_3 ou espace T_3) a été établi et nous avons déterminé par ailleurs l'espace sodium $\left(R_{Na} = \frac{^{24}\text{Na/mg tissu}}{^{24}\text{Na/mg plasma}}\right)$ à des fins de comparaison.

La Fig. 5 illustre la rapidité de la disparition de T_3 plasmatique, nettement plus grande que celle de ST_3. L'essentiel des résultats obtenus sur les organes a été rassemblé dans la Fig. 6 et le Tableau I. L'examen de ceux-ci montre que le rein seul concentre assez activement ST_3 ($R_I > R_{Na}$), contrairement à ce qui a lieu pour T_3, en particulier dans le foie. Bien que le pourcentage de la dose totale de ST_3 fixée par le foie et les muscles soit élevé, ces organes ne drainent pas ST_3 plasmatique vers les espaces extra-cellulaires comme ils le font pour T_3. La conséquence physiologique de ce fait sera discutée plus bas.

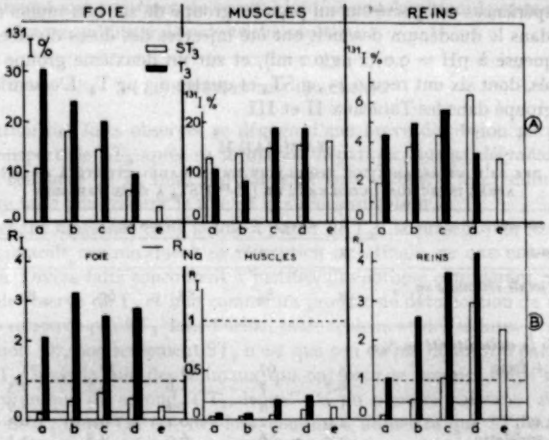

Fig. 6. A. Radioactivité totale (R') du foie, des muscles et des reins, exprimée en % de la dose administrée et mesurée à des temps différents après l'injection intraveineuse aux rats de $[3'-^{131}I]ST_3$ —colonnes blanches—et après l'administration de $[3'-^{131}I]T_3$—colonnes noires—à la dose de 0.6 µg/100 g. B. Valeurs de $R_I = {}^{131}I$/mg tissu/${}^{131}I$/mg plasma, pour les mêmes organes, dans des conditions expérimentales identiques. (A titre comparatif, la valeur de l'espace sodium des tissus considérés est indiquée par le trait plein horizontal.) Temps en abscisses: a = après 5 min; b = après 20 min; c = après 60 min; d = après 180 min; e = après 360 min.

TABLEAU I

VALEURS MOYENNES DE $R_I = {}^{131}I$/MG DE TISSU/${}^{131}I$/MG DE PLASMA A DIFFÉRENTS TEMPS APRÈS L'INJECTION INTRAVEINEUSE DE $[3'-^{131}I]ST_3$

Valeurs affectées des erreurs quadratiques moyennes correspondantes. ($R_{Na} = {}^{24}$NaCl/mg de tissu/^{24}NaCl/mg de plasma = espace sodium du tissu considéré du rat.)

Temps (min)	*Intestin grêle avec son contenu*	*Poumons*	*Coeur*	*Surrénales*	*Rate*
5	0.03 ± 0.004	0.13 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.04 ± 0.01
20	0.22 ± 0.04	0.42 ± 0.11	0.15 ± 0.02	0.24 ± 0.01	0.12 ± 0.01
60	0.48 ± 0.20	0.34 ± 0.02	0.20 ± 0.02	0.15 ± 0.07	0.12 ± 0.01
180	1.27 ± 0.40	0.27 ± 0.04	0.22 ± 0.03	0.21 ± 0.10	0.18 ± 0.04
360	0.86 ± 0.20	0.45 ± 0.07	0.25 ± 0.03	0.27 ± 0.09	0.15 ± 0.03
R_{Na}	0.35		0.36	0.48	

Métabolisme de ST_3 et de T_3 injectés dans le duodénum: L'excrétion biliaire de ST_3 administré par voie sanguine est moins importante que celle de T_3 et de ses métabolites hépatiques, mais la radioactivité fécale est pratiquement la même à des temps successifs après l'injection de l'un ou de l'autre corps. Il y avait, dès lors, lieu d'étudier leur résorption intestinale.

Les expériences ont porté sur un premier groupe de six rats munis d'une fistule biliaire et dans le duodénum desquels ont été injectées des doses diverses de ST_3 en solution aqueuse à pH = 9.0 (1 μg/0.1 ml), et sur un deuxième groupe de dix rats non fistulisés, dont six ont reçu 0.25 μg ST_3 et quatre 0.3 μg T_3. L'essentiel des résultats a été groupé dans les Tableaux II et III.

TABLEAU II

EXCRÉTION DES 24 H ET RÉPARTITION TISSULAIRE DE LA RADIOACTIVITÉ À LA 24ème HEURE, APRÈS INJECTION DUODÉNALE DE $[3'\text{-}^{131}I]ST_3$ À DOSE VARIABLE

ST_3: dose injectée par 100 g	*0.2 μg*		*0.5 μg*		*2.5 μg*	
Rats thyroïdectomisés	*1*	*2*	*3*	*4*	*5*	*6*
Radioactivité totale retrouvée en % de la dose	91	84	88	69	80	87
Repartition de la radioactivité en %:						
Intestins + fèces	73	67	84	70	72	74
Bile	6.7	7	1.6	5.7	7.5	9
Urine	1	4	1.4	7.7	2.9	2.2
Plasma (1 ml)	1.9	2	0.2	0.2	0.3	0.5
Muscle + squelette	4.2	6	6.9	3.8	3.5	2.5
Foie	2.3	2.1	0.7	0.5	0.7	0.9
Reins	0.8	0.4	0.4	0.1	0.6	0.5
Peau + organes restants	10.1	11.5	4.8	12	12.5	10.4

TABLEAU III

EXCRÉTION DES TROIS JOURS ET RÉPARTITION TISSULAIRE DE LA RADIOACTIVITÉ AU TROISIÈME JOUR, APRÈS INJECTION DUODÉNALE DE $[3'\text{-}^{131}I]ST_3$ ET DE $[3'\text{-}^{131}I]T_3$

Valeurs moyennes affectées de leur erreur quadratique et exprimées en pour cent de la dose administrée. P: coefficient de probabilité d'après le test "t" de Student.

Traitement et nombre d'animaux	*ST_3 0.25 μg/100 g (6 rats)*	*T_3 0.3 μg/100 g (4 rats)*	*Coefficient P*
Radioactivité totale effectivement retrouvée	87 ± 2.6	99 ± 7.1	0.1 ± 0.05
Répartition en %:			
Urine	15.30 ± 2.6	38.40 ± 3.4	0.01
Fèces	56.20 ± 8.7	58.10 ± 2.6	—
Tube digestif	16.70 ± 6.1	0.80 ± 0.03	0.10–0.05
Peau	8.80 ± 2.1	2.0 ± 1.4	0.05–0.02
Muscles et Squelette	2.46 ± 0.66	0.34 ± 0.08	0.05–0.02
Foie	0.30 ± 0.02	0.32 ± 0.07	—
Rein	0.16 ± 0.04	0.03 ± 0.01	0.10–0.05
Plasma (1 ml)	0.08 ± 0.03	0.01 ± 0.003	0.10
Fraction résorbée en 3 jours	27 ± 3.9	41 ± 2.7	0.05–0.02

La résorption intestinale de ST_3 est notablement moins rapide et moins intense que celle de T_3 et, dans un cas comme dans l'autre, la radioactivité retrouvée dans l'urine est presque entièrement due à la présence d'iodures, alors que celle des fèces est comprise dans des combinaisons organiques. En effet, 90–95 % d'^{131}I urinaire présentent les caractères électrophorétiques des iodures, alors que 90–98 % d'^{131}I

des fèces se fixent aux protéines du sérum ajouté à une émulsion de ceux-ci et précipitent avec elles par addition d'acide trichloracétique.

DISCUSSION DES RESULTATS

La signification des faits observés se dégage d'une brève discussion portant sur le cycle de transport de ST_3 après sa formation hépatique, sur sa dégradation et sur l'utilisation cellulaire de T_3 véhiculée par le plasma à l'état d'ester sulfurique. Elle mérite d'être faite pour définir le rôle de la sulfoconjugaison de T_3.

ST_3 prenant naissance dans le foie à partir de T_3[1], la présence de ce corps dans le plasma apparaît comme due à sa résorption intestinale, ce que nos expériences ont contrôlé. Divers faits concourent à justifier l'hypothèse considérant ST_3 comme une forme de réserve de T_3 et non comme un produit de détoxication de celle-ci. En effet, on ne retrouve pas ST_3 dans l'urine, mais seulement des iodures provenant de sa dégradation. Or, non seulement ST_3 n'est que peu ou pas concentré par les cellules qui fixent T_3, mais la bile des animaux qui ont reçu le premier corps renferme le dérivé glycuronique du second, GT_3, lequel n'a pu prendre naissance dans le foie qu'à partir de T_3 libre. On est, dès lors, conduit à admettre que ST_3 est hydrolysé par une arylsulfatase tissulaire et que T_3 libéré est alors métabolisé. S'il en est ainsi, ST_3 constitue une réserve circulante de T_3, relativement inerte en elle-même, mais susceptible de donner naissance à l'hormone, qui serait immédiatement utilisée.

La différence des rôles respectifs de la glycuroconjugaison et de la sulfoconjugaison de T_3 est manifeste. GT_3 est, en effet, rapidement hydrolysé par la β-glycuronidase des bactéries intestinales et T_3 qu'il renferme participe à la circulation entérohépatique de l'hormone. T_3 de GT_3 est, dès son entrée dans la circulation sanguine, utilisée par les cellules, au même titre que l'hormone, sécrétée par la glande thyroïde et non conjuguée par le foie. ST_3, au contraire, n'est que peu dédoublé par une arylsulfatase intestinale et est en partie résorbé tel quel. N'étant pas concentré dans les cellules, il ne paraît céder T_3 à celles-ci qu'au fur et à mesure de son hydrolyse, réalisée par une arylsulfatase tissulaire. GT_3 est donc une sorte d'intermédiaire biliaire dans la circulation entérohépatique de T_3, ne pouvant pas jouer le rôle d'une réserve de celle-ci et normalement absent du plasma[5], tandis que ce rôle paraît dévolu à ST_3. A cet égard, la sulfoconjugaison de T_3 mérite d'être rapprochée de celle d'autres hormones phénoliques[6], processus sur lequel repose la constitution d'une réserve circulante de ces hormones.

Les faits établis apparaissent comme d'autant plus significatifs que le foie réalise préférentiellement la glycuroconjugaison de la L-thyroxine (T_4) et la sulfoconjugaison de son homologue triiodé T_3[7], dont l'activité sur le métabolisme cellulaire est beaucoup plus forte.

RÉSUMÉ

1. Les métabolismes comparés de la 3,5,3'-triiodothyronine (T_3) et de son sulfoconjugué (ST_3) ont été étudiés chez le Rat thyroïdectomisé. Le premier de ces corps est dégradé plus rapidement que le second, que les cellules concentrent beaucoup moins intensément.

2. Les caractères du métabolisme de ST_3, entre autres la formation à ses dépens du glycuroconjugué de T_3 par le foie et l'excrétion d'iodures après son injection, sa

présence dans le plasma et son absence dans l'urine, permettent d'envisager qu'il est hydrolysé en partie dans les tissus par une arylsulfatase et que son constituant hormonal est désiodé.

3. Le rôle probable de ST_3 est celui d'une forme de réserve de T_3 accessible aux cellules après hydrolyse de la liaison ester.

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METABOLISM OF D-[1-^{14}C]- AND D-[6-^{14}C]GLUCURONOLACTONE BY THE RIPENING STRAWBERRY[*]

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SUMMARY

D-[1-^{14}C] and D-[6-^{14}C]glucuronolactone is metabolized by the detached ripening strawberry fruit to several six carbon acids including gulonic acid, L-ascorbic acid, D-galacturonic acid, and D-glucuronic acid. In addition, one of the acid intermediates is decarboxylated to yield free D-xylose from carbons 1 through 5 and CO_2 from carbon 6 of D-glucuronolactone. A portion of carbons 1 through 5 is also utilized in sucrose synthesis and in pentose residues of cell-wall polysaccharides. The ^{14}C distribution patterns and specific activities of the identified constituents suggest that two pathways of glucuronolactone utilization reside in the strawberry. The evidence for these pathways is discussed.

INTRODUCTION

Tracer studies of the over-all conversion of glucose[***] to ascorbic acid by the detached, ripening, strawberry fruit[2,3] and by the etiolated, germinating, cress seedling[4] have revealed that the six carbon chain is conserved and that C-1 of glucose is oxidized forming the carboxyl (C-1) carbon of ascorbic acid. The utilization of glucose for ascorbic acid formation in the strawberry appeared to follow normal processes of hexose phosphate metabolism[5-7].

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[*] A preliminary report of this work has been published[1].

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[***] The prefixes D and L are omitted in all instances where the naturally-occurring isomer is mentioned and where there will be no question of configuration.

We have, however, confirmed the existence of a path of conversion from glucuronolactone (GL) to ascorbic acid in the strawberry that corresponds to the stereochemical requirements of the scheme described by ISHERWOOD, CHEN AND MAPSON[8]. [1-^{14}C]GL was converted, in part, to ascorbic acid labeled almost exclusively in C-6[3]. This finding has prompted a more detailed study of the metabolism of GL.

EXPERIMENTAL

Materials and methods

[1-^{14}C]GL was synthesized chemically from [1-^{14}C]glucose[9]. [6-^{14}C]GL was prepared from [6-^{14}C]sodium glucuronate obtained from Dr. H. S. ISBELL, National Bureau of Standards, Washington, D. C. Both lactones were purified just prior to use by paper chromatography.

Field grown strawberries (*Fragaria*, variety Shasta) were harvested in the early white stage of ripening on July 21, 1958. The stems were cut under water and kept thus until placed in the radioactive solutions. After the radioactive material had been taken up by the berries (0.4 ml), water was added to keep the cut stems immersed until the respiration study was completed (41 h). At the end of this time, both berries were red and had gained 0.6 g. The respired CO_2 was trapped in *N* NaOH.

Separation of berry constituents and degradation procedures

The methods of separation and isolation of the carbohydrate constituents have been described previously[2,5,7]. A slight modification was introduced in the method by which ascorbic acid was eluted from the Dowex 1 (formate) exchange column in order to reduce the contamination of ascorbic acid with other labeled acidic products of GL metabolism[2,7]. The berry acids from the ethanol-soluble fraction that remained on the Dowex 1 (formate) (1 × 10 cm, 200–400 mesh, medium porosity) were eluted with a gradient of 0.02 → 0.06 *N* formic acid that consisted of a reservoir of 500 ml of 0.06 *N* formic acid which flowed through a mixing chamber containing 200 ml of 0.02 *N* formic acid onto the top of the resin column. After 500 ml of eluate had been collected in 5-ml fractions, 200 ml of 3 *N* formic acid was added to the reservoir and an additional 200 ml of eluate collected. Finally, 35 ml of 6 *N* formic acid was added directly to the top of the column and collection of fractions continued until the column was dry.

Galacturonic acid from the fungal pectinase hydrolysate of the 70% ethanol-insoluble portion of the strawberries was also recovered using the anion exchange column and formic acid gradient just described.

All carbohydrate constituents scheduled for degradation were first recrystallized until there was no further change in radioactivity. The methods employed in degradation and counting have been described previously[2,5-7].

RESULTS

Distribution of ^{14}C among the berry fractions

A plot of the ^{14}C accumulation in respired CO_2 during the uptake and metabolism of labeled GL is shown in Fig. 1. ^{14}C from [6-^{14}C]GL appeared almost immediately

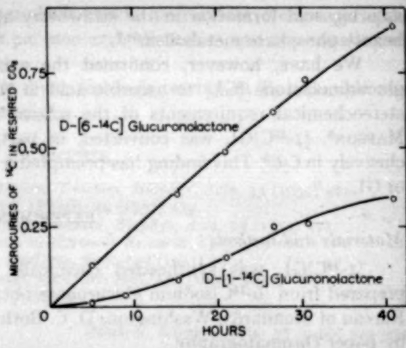

Fig. 1. A plot of ^{14}C accumulation in the respired CO$_2$ as a function of time. The ^{14}C values given for the [1-^{14}C]glucuronolactone have been reduced by a factor of 0.76 in order to permit direct comparison of respired activities between the two experiments.

TABLE I

DISTRIBUTION OF RADIOACTIVITY IN LABELED STRAWBERRIES

		D-glucuronolactone	
		[1-^{14}C]	[6-^{14}C]
		μC	μC
^{14}C	Administered	3.8 (1.46 mg)	2.9 (2.84 mg)
^{14}C	Recovered		
	Respired CO$_2$	0.44	0.87
	Berry, ethanol-soluble	0.90	0.91
	Stem and calyx, ethanol-soluble	0.82	0.51
	Pectinase solubilized	0.21	0.12
^{14}C	Unaccounted for*	1.43	0.5

* Seeds, unhydrolyzed cell wall material and related insolubles.

TABLE II

DISTRIBUTION OF RADIOACTIVITY IN ETHANOL-SOLUBLE PORTION OF LABELED STRAWBERRIES

		D-glucuronolactone	
		[1-^{14}C]	[6-^{14}C]
		mμC	mμC
Activity added to Dowex 1		900	910
Activity of acidic pooled fractions*	A (0– 30 ml)	8	4
	B (90–215 ml)	150	170
	C (215–340 ml)	51	34
	D (340–450 ml)	14	31
	E (510–615 ml)	89	51
	F (640–700 ml)	32	17
Activity of neutral effluent		440	180
Activity of sucrose and xylose only		240	negligible
Activity lost during separations		120	420

* Pooled fractions corresponding to the various radioactive peaks eluted from the Dowex 1 (formate) resin with the dilute formic acid gradient. The successive volume increments are indicated in parentheses.

and continued to accumulate at a linear rate during the respiration. When the experiment ended, 30 % of the ^{14}C from [6-^{14}C]GL had been converted to CO_2. In contrast to this observation, [1-^{14}C]GL was metabolized to $^{14}CO_2$ by the strawberry only after a lag period. Less than 12 % of the administered ^{14}C appeared in the CO_2. [1-^{14}C]GL resembled [1-^{14}C]glucose and [1-^{14}C]xylose in this respect. The preferential decarboxylation of C-6 of GL has also been observed in corn coleoptiles by SLATER AND BEEVERS[10] and in rat kidney preparations by RABINOWITZ AND SALL[11].

A crude accounting of the ^{14}C distribution in the berries is presented in Table I. The ^{14}C remaining in the 70 % ethanol-insoluble portion of the berries after treatment with fungal pectinase has not been examined but the work of NEISH[12] has already demonstrated that considerable ^{14}C from [1-^{14}C]GL is incorporated into cell wall xylan and cellulose.

The distribution of activity among the various crude fractions of the ethanol-soluble portion of each berry after removal of the stem and calyx is given in Table II. About a third of this activity remained on the anion exchange column after it had been thoroughly washed with water. The neutral effluent which had previously been shown to consist primarily of free sugars (sucrose, glucose, fructose, xylose, and a trace of ribose[5]) also contained, in the present experiments, some labeled GL. Very little ^{14}C appeared in the free sugars from the [6-^{14}C]GL-labeled berry and the 180 mμC found in the neutral effluent was almost entirely due to GL. The neutral effluent from the alcohol solubles of the [1-^{14}C]GL-labeled berry contained, in addition to [^{14}C]GL, considerable activity in the free xylose and sucrose confirming our earlier report of the preferential labeling of these sugars[3].

Elution of the activity retained by the anion exchange resin resulted in several radioactive peaks. The fractions corresponding to these peaks were pooled as described in Table II. Elution patterns from [1-^{14}C]GL- and [6-^{14}C]GL-labeled berries were similar.

Pooled fractions "B" contained one major radioactive component and at least six minor components. The major component could be lactonized and did not react with aniline–trichloroacetic acid spray reagent (an aldose test). When chromatogrammed on paper in three different solvent systems, the lactone of the major component migrated identically with L-gulonolactone. Comparisons were made with D-mannonolactone, D-altronolactone, L-galactonolactone, L-idonolactone, and D-gluconolactone in ethyl acetate–acetic acid–water (3:1:3)[13], ethyl acetate–pyridine–water (8:2:1)[14] and 2,2-dimethyl-1-propanol–water–1-propanol–ethanol (4:2:1.3:0.5)[15]. In all of these systems, gulonolactone was the slowest moving aldonolactone tested.

Pooled fractions "C" included ascorbic acid which was recovered by dilution with unlabeled ascorbic acid followed by successive crystallizations from glacial acetic acid until the activity remained constant. Less than one-half of the activity in "C" could be ascribed to ascorbic acid.

Pooled fractions "D" were eluted in the fractions that characterized glucuronic acid in separate control runs. It gave an aniline–trichloroacetic acid spot test similar to glucuronic acid and probably represented the non-metabolized GL that was hydrolyzed to free acid by the berry. Pooled fractions "E" and "F" have not been investigated as yet.

Table II also records the portion of ^{14}C unaccounted for after anion exchange

TABLE III

DISTRIBUTION OF RADIOACTIVITY IN PECTINASE-HYDROLYZED SOLUBLES OF LABELED STRAWBERRIES

	D-glucuronolactone	
	[1-^{14}C]	*[6-^{14}C]*
	mμC	*mμC*
Activity added to Dowex 1	194	114
Activity in galacturonic peak	73	80
Activity in other peaks (combined)	24	11
Activity of neutral effluent	94	2
Activity lost (unaccounted for)	3	21

resin treatment of the ethanol solubles. In the case of the berry labeled with [6-^{14}C]GL this loss represented almost one-half of the added activity. Presumably, this loss of activity was due to decarboxylation of unknown, labile, acidic components. Further work on the nature of this phenomenon is planned.

The distribution of radioactivity in the soluble pectinase hydrolysate of the 70 % ethanol insoluble portion of each berry is given in Table III. A major portion of the activity retained on the anion exchange column was located in galacturonic acid. A smaller radioactive peak of glucuronic acid followed the galacturonic acid in both experiments. Galacturonic acid was recovered from the pooled fractions as its sodium calcium salt[16].

A significant amount of ^{14}C appeared in the neutral effluent only when [1-^{14}C]GL was the source of label. Paper chromatography followed by radioautography[7] revealed just two radioactive components, arabinose and xylose. Neither glucose nor galactose residues were labeled although, quantitatively, these hexoses accounted for two-thirds of the sugar residues in the pectinase-hydrolysate.

Labeling patterns of ascorbic acid, galacturonic acid and sugar constituents

The activity and distribution of ^{14}C in the carbon chains of ascorbic acid and galacturonic acid residues are given in Table IV. Earlier attempts[3,7] to demonstrate

TABLE IV

RADIOACTIVITY AND SITE OF LABELING IN ASCORBIC ACID AND GALACTURONIC ACID FROM [1-^{14}C]- AND [6-^{14}C]GLUCURONOLACTONE LABELED STRAWBERRIES

	[1-^{14}C]glucuronolactone			*[6-^{14}C]glucuronolactone*	
	Ascorbic acid	*Galacturonic acid*		*Ascorbic acid*	*Galacturonic acid*
Recovered**, mg	6.1	31*	26.1	5.5	33.4
Dilution factor	21.6	1.4	5.8	27.7	4.7
Total activity, mμC	30	46	77	32	74
Specific activity, mμC/mg carbon	12	40	8	14	6
	Per cent of total activity				
Activity, carbon 1	—	99	99	95	—
Activity, carbon 6	99	—	1	2	99

* This column contains data on galacturonic acid recovered from a berry used in an earlier experiment[3].

** Based on assay before carrier dilution.

a conversion of ^{14}C from [6-^{14}C]GL to ascorbic acid were unsuccessful since the anion exchange procedure employed at that time could not separate ascorbic acid from the radioactive aldonic acid found in pooled fractions "B" in the present study.

The results in Table IV provide isotopic evidence for the over-all conversion of GL to ascorbic acid with inversion of the intact carbon chain as originally described by ISHERWOOD, CHEN AND MAPSON[8]. Our earlier demonstration of the conversion of [1-^{14}C]GL to [6-^{14}C]ascorbic acid[3] is confirmed in the present experiment and substantiated by the [6-^{14}C]GL to [1-^{14}C]ascorbic acid conversion. Both [1-^{14}C]GL and [6-^{14}C]GL were converted to galacturonic acid by an epimerization of C-4.

Very little ^{14}C from [6-^{14}C]GL was incorporated into the free sugars or the pectinase-solubilized cell wall polysaccharides. [1-^{14}C]GL was converted by the strawberry to free xylose and sucrose, and to arabinose and xylose residues in the cell-wall polysaccharides. Table V gives the labeling patterns from the present experiments as well as unreported data on the sugars of the previously reported [1-^{14}C]GL experiment[3]. Free xylose had the highest specific activity, 10 and 31 % of the administered label (1050 mμC/mg carbon). A portion of free xylose from the previous experiment was converted to 2:4, 3:5 dibenzilidene xylose dimethyl acetal, a reaction highly specific for xylose[17], with no change in radioactivity in the product. Another portion of this free xylose was degraded microbiologically with D-xylose adapted *Lactobacillus plantarum*. 99 % of the ^{14}C appeared in the acetic acid fragment (C-(1 + 2)) which chemical degradation revealed to be labeled only in the methyl carbon (C-1 of the original xylose). These data have been interpreted as evidence that the major free pentose product from [1-^{14}C]GL was D-xylose.

TABLE V

RADIOACTIVITY AND SITE OF LABELING IN SUCROSE-DERIVED GLUCOSE AND PENTOSES FROM [1-^{14}C]GLUCURONOLACTONE LABELED STRAWBERRIES

		Sucrose-derived glucose	*Sucrose-derived glucose*	*Free xylose*	*Free xylose*	*Pectinase-hydrolysed*			
						Xylose		*Arabinose*	
		*		*		*		*	
Recovered**, mg		2.2	7.1	6	3.6	0.8	1.1	1.6	6.1
Dilution factor		46.4	15.2	61	57.3	175	93.7	89	17.9
Total activity, mμC		19	20	806	144	46	11	46	39
Specific activity, mμC/mg carbon		21	7	330	100	143	25	72	16
		Per cent of total activity							
Activity carbon	1	41.5	49	99	84	92	90	100	99
	2	4	4.5		11	8	8		
	3	13	15						
	4	12	9.5						
	5	5.5	2		5		2		
	6	24	20						

[*] These columns contain data on sugars recovered from a berry used in an earlier experiment[3].
[**] Based on assay before carrier dilution.

The glucose and fructose moieties of sucrose were equally labeled. Both their specific and total activities were considerably less than that of the free xylose. The activities and distribution patterns in the carbon chains of the glucose moieties are given in Table V. C-1 contained the most ^{14}C. C-3, C-4, and C-6 also contained significant amounts of label. The appearance of ^{14}C in C-1 and C-3 is particularly significant since it suggests that a pentose labeled in C-1 was the precursor[18]. Redistribution from C-1 and C-3 into C-6 and C-4 respectively corresponds to the normal pattern of label redistribution among hexose phosphate-derived constituents of the strawberry[2,5,6] as well as those of other plants. A previous study of the metabolism of [1-^{14}C]xylose by the strawberry[6] revealed that sucrose was the primary free sugar product that became labeled. It appears reasonable to assume that in the conversion of [1-^{14}C]GL to ^{14}C-sucrose, a [1-^{14}C]pentose precursor was the principle intermediate.

Table V also records the activities and patterns of xylose and arabinose residues that were isolated from the pectinase hydrolysate of the 70% ethanol-insoluble portion of the [1-^{14}C]GL-labeled berries. Both pentose residues had lower specific activities than did free xylose. Essentially no redistribution of ^{14}C occurred in the arabinose, C-1 retained all the activity. In xylose residues, between 8 to 10% of the ^{14}C incorporated was in carbons other than C-1. These patterns differ from those obtained when [1-^{14}C]xylose was the source of label[6]. It is entirely possible that [1-^{14}C]xylose administered through the vascular tissues of the berry is utilized quite differently in polysaccharide synthesis from [1-^{14}C]pentose generated from [1-^{14}C]GL *in situ*.

DISCUSSION

The discovery that free D-xylose was a product and that it corresponded to the first five carbons, while C-6 of GL was lost as CO_2 implied a direct decarboxylation of GL. ALTERMATT AND NEISH[19] and NEISH[12] have already discussed this possibility in terms of uridine diphosphate derivatives in the course of their tracer studies of cellulose and xylan synthesis in wheat seedlings. HASSID *et al.*[20–22] have experimentally demonstrated the conversion of glucuronic acid to the uridine diphosphate derivatives of galacturonic acid, xylose and arabinose by plant extracts. Their findings explain the observed conversion of GL to cell wall polysaccharides in the strawberry. If the same path of conversion is responsible for free xylose formation, then a hydrolytic mechanism is also necessary although experimental evidence is still lacking. Alternatively, free xylose could be formed by non-phosphorylative reactions related to the production of gulonic acid and ascorbic acid during GL utilization by the berry. This was discussed in a previous paper[3].

The exact nature of the non-phosphorylative conversion of GL to gulonic acid and to ascorbic acid and, possibly, to xylose is not fully understood. ISHERWOOD, CHEN AND MAPSON[8] proposed a direct reduction of GL to L-gulonolactone which was then oxidized to an ascorbic acid precursor. Recent studies on the metabolism of GL and glucuronic acid by certain bacteria[23–25] have revealed that the first step is an isomerization to D-fructuronic acid followed by a reduction of this compound to D-mannonic acid[23]. Although the corresponding reaction has not yet been reported to be present in plants, it provides a reasonable explanation for the appearance of gulonic acid (the C-2 epimer of mannonic acid) which could be obtained from fructuronic acid by a stereospecific reduction. Assuming that fructuronic acid, and not gulonic acid, is the intermediate that is oxidized at C-4 (corresponding to C-3 of gulonic acid), then the product would be 3,5-diketogulonic acid. This diketo compound could be decarboxylated as a beta-keto acid to form a pentose directly, or it could conceivably enolize at the beta-keto function to form the 5-keto analog of ascorbic acid.

3,5-diketogulonic acid differs from 3,5-diketogluconic acid only in the configuration about C-2. Enolization of the C-3 keto function would destroy this difference, providing a common intermediate from GL *via* fructuronic acid and from glucose *via* 3-keto-6-phosphogluconic acid[3,7]. A stereospecific reduction of C-5 to L-ascorbic acid would represent the final step. Such a scheme would accommodate all of the tracer patterns obtained with labeled glucose or GL.

NOTE ADDED IN PROOF

Positive characterization of the major component of peak "B" as L-gulonic acid has recently been completed and reported elsewhere[26].

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Short Communications

Potentiometric iodometric determination of plasmalogen

Difficulties in detecting the visual end point in the iodination procedure of Rapport and Lerner[1] for the determination of plasmalogen were noted in titrating highly colored lipid extracts. The end point also could not be determined reproducibly with colorless samples containing less than 2 μmoles plasmalogen. In addition it was desired to follow the concentration of plasmalogen and other enol ethers in the course of reactions with colored thiols and thiol acids. For these reasons the iodination procedure has been adapted for potentiometric end-point detection.

The advantages of the potentiometric method are: (a) Higher accuracy and precision especially in colored samples. (b) Smaller samples may be used. (c) Elimination of the two-phase system during titration and therefore elimination of the need for vigorous agitation by nitrogen bubbling which we have found causes iodine losses.

Fresh rat tissues and beef adrenals obtained directly from the slaughter house (trimmed and dissected into cortex and medulla at 0–4°) were extracted immediately by the method of Folch, Lees and Sloane-Stanley[2]. These washed total-lipid extracts were taken to dryness *in vacuo* at 40°, redissolved in $CHCl_3$–MeOH (2 : 1, v/v), filtered and made up to volume to contain 1–3 μmoles plasmalogen/ml. To 1.00 ml of this solution in a 10-ml beaker is added 1.00 ml 0.01 N aq. I_2. This is mixed by swirling for 30 sec at room temperature and then diluted with 3.0 ml 95 % ethanol. The micro platinum and calomel electrodes of a standard line-operated pH meter (Beckman model H-2 was used in this study) are immersed in the solution and titration of the excess I_2 is carried out rapidly with 0.1 N $Na_2S_2O_3$ supplied from a 0.1-ml ultramicroburet. Good agitation by a magnetic stirrer is essential.

Beginning at about 0.01 ml before the end point the titrant is added in 0.001 ml increments and the e.m.f. is recorded after each increment. The end point is determined graphically in the usual way from the S-shaped titration curve, the end point occurring at about 150 mV. In these very dilute I_2 solutions the visual end point occurs approximately 10 % before the true end point determined potentiometrically. The potentiometric end point is determined to 0.0001 ml and the absolute precision of the method is $\pm$ 0.0002 ml; therefore, 0.5 μmole can be determined with a precision of $\pm$ 2 % and 3 μmoles with a precision of $\pm$ 0.3 %. These values vary from one tissue to another. For all tissues studied (see Table I) with varying plasmalogen concentrations both in the tissue and the final lipid solution, the average precision was $\pm$ 1.3 %; however, plasmalogen in a sheep-brain extract was determined at the 1 μmole level with a precision of $\pm$ 0.3 %.

All tissues studied were also analyzed for long-chain fatty aldehydes by the *p*-nitrophenylhydrazone colorimetric procedure[3]. The average value for all tissues, exclusive of rat liver, of the molar ratio of I_2 to *p*-nitrophenylhydrazone is 0.96. This is compared to the findings of Rapport and Lerner[1] who obtained 1.08 for this ratio.

TABLE I

PLASMALOGEN CONTENT OF RAT TISSUES AND BEEF ADRENALS

The water content was obtained by drying 500 mg tissue samples for 20 h at 70° *in vacuo*. Lipid weights were obtained by drying aliquots of the reconstituted $CHCl_3$–MeOH extract under the same conditions. Lipid P was estimated by the method of FISKE AND SUBBAROW[4]. The plasmalogen values, reported as µmole/g dry wt., are averages of at least 4 determinations except in the case of the *p*-nitrophenylhydrazone values for beef adrenals where only two values were averaged. The plasmalogen as per cent of total lipid was calculated assuming 750 for the molecular weight of plasmalogen.

Tissue	*No. of animals*	*Water %*	*Lipid % dry wt.*	*Lipid P µmole/g dry wt.*	*Plasmalogen (I_2 titration) µmole/g dry wt.*	*Plasmalogen (as p-nitrophenylhydrazone)*	*Plasmalogen % of total lipid*	*µmole I_2 / µmole hydrazone*
Rat brain	7	78.7	40.0		55.2 ± 0.6	52.8 ±0.9	10.3	1.05
Rat brain	10	78.8	34.8		48.9 ± 0.8	48.9 ± 2.0	10.6	1.00
Rat brain	10	78.9	34.8		51.9 ± 0.9	48.3 ± 2.7	11.2	1.07
Rat heart	8	79.1	18.0		11.7 ± 0.2	12.4 ± 0.05	4.9	0.94
Rat heart	10	78.0	16.0		12.2 ± 0.1	13.8 ± 0.3	5.8	0.88
Rat liver	2	68.8	16.7		2.13 ± 0.02	2.96 ± 0.10	0.96	0.72
Rat liver	5	69.4	15.0		2.19 ± 0.07	3.13 ± 0.20	1.1	0.70
Rat lung	8	80.0	20.0		14.4 ± 0.1	17.4 ± 1.4	5.4	0.83
Rat lung	10	80.2	17.9		15.3 ± 0.1	15.0 ± 1.9	6.4	1.02
Rat lung	10	79.5	19.0		17.1 ± 0.5	16.2 ± 0.6	6.8	1.06
Rat kidney	8	78.1	27.1		12.2 ± 0.15	13.0 ± 0.3	3.4	0.94
Rat kidney	10	77.1	23.5		11.0 ± 0.2	10.5 ± 1.8	3.5	1.04
Rat kidney	10	77.6	31.1		12.5 ± 0.5	14.2 ± 0.8	3.0	0.88
Rat skeletal muscle	4	76.0	17.5		5.28 ± 0.05	5.9 ± 0.3	2.3	0.90
Rat skeletal muscle	4	75.5	17.3		5.36 ± 0.13	6.17 ± 0.16	2.3	0.87
Beef adrenal cortex	3	78.2	20.4	178	22.3 ± 0.5	23.3	8.2	0.96
Beef adrenal medulla	6	79.1	14.9	141	25.4 ± 1.2	24.5	12.8	1.04
Beef whole adrenal	1	78.2	21.4	195	25.2 ± 0.9	27.5	8.8	0.92

The discrepancy between these two ratios may be partially accounted for by the general differences in technique employed in the two iodination methods.

The large reproducible difference between the two analytical methods for plasmalogen in rat liver is unexplained. RAPPORT AND LERNER also found that rat liver gave lower molar ratios of iodine to *p*-nitrophenyl-hydrazone than the other rat tissues studied. We believe this observation has significance in regard to plasmalogen metabolism and is under further investigation.

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V.
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Thermal denaturation of deoxyribosenucleic acid isolated from a thermophile

In the course of an investigation of the thermal stability of highly polymerized deoxyribosenucleic acids, isolated from various microorganisms, it was found that the denaturation of the DNA was related to the overall mole percent composition of guanine plus cytosine in the sample[1]. It was thought of interest to study the heat denaturation of polymerized DNA isolated from a thermophile grown at an elevated temperature. *Bacillus stearothermophilus* was grown in the basal medium of BAKER *et al.*[2] supplemented with 0.5 % acid-hydrolyzed casein in shallow players at 55° for 16 h (cells mostly in the vegetative state). The cells were harvested, washed, lyophilized and send to us by Dr. BAKER[2]. The guanine plus cytosine content of the DNA of *B. stearothermophilus* has been found to average 46.7 %[2,3]. The denaturation of *Escherichia coli* (K-12) DNA, which has a base composition (50 % guanine plus cytosine) similar to that of *B. stearothermophilus*, was studied simultaneously for comparison. DNA was isolated from both organisms by first disrupting the vegetative cells with sodium lauryl sulfate followed by deproteinization with chloroform and isoamyl alcohol*. The sedimentation coefficient, $S_{20,w}$, measured at infinite dilution was found to be 18.3 for *B. stearothermophilus* and 33.4 for *E. coli*. Thermal denaturation of the DNA was studied by determining the absorbance–temperature profile n a thermostated Beckman Spectrophotometer Model DU at 260 mμ. DNA dissolved in 0.15 *M* NaCl plus 0.015 *M* sodium citrate, pH 7, was placed in glass-stoppered

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Abbreviation: DNA, deoxyribosenucleic acid.

* A detailed description of this method will be published shortly.

cuvettes and the absorbance recorded at each successive elevated temperature after the solutions reached temperature equilibrium.

The absorbance-temperature profiles are shown in Fig. 1. It is apparent from the narrow temperature range in which the absorbance rises that the DNA's isolated from both *B. stearothermophilus* and *E. coli* are native[4,5]. The temperature at the midpoint of the absorbance rise, T_m, is 87.5° for *B. stearothermophilus* and 90° for *E. coli*. The value of the T_m for *B. stearothermophilus* predicted from the relationship between the base composition and T_m[1] is in very close agreement, namely 88°. The lower molecular weight of the DNA from the thermophile could not be expected to have a significant influence on its T_m since it has been shown that the sonic degradation of calf thymus and *Diplococcus pneumonia* DNA's to molecular weights of the order of 600,000 ($S_{20,w}$ about 9) has very little influence on their T_m.

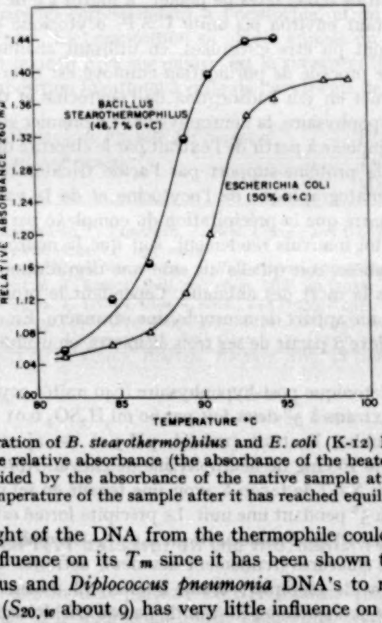

Fig. 1. Thermal denaturation of *B. stearothermophilus* and *E. coli* (K-12) DNA in 0.15 M NaCl + 0.015 M Na citrate. The relative absorbance (the absorbance of the heated sample, corrected for thermal expansion, divided by the absorbance of the native sample at 25°) is plotted against the temperature of the sample after it has reached equilibrium.

Thus the thermal stability of the DNA from *B. stearothermophilus* is not unusual: the high temperature adaptation of this bacterium appears to arise from its proteins, some of which exhibit high heat resistance[7,8].

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Isolement de l'ocytocine du poulet

L'ocytocine a été isolée à partir de la post-hypophyse de différentes espèces de mammifères: boeuf[1,2], porc[3], cheval[4], mouton[5] et homme[6]. Dans tous les cas, l'hormone obtenue possédait la même composition chimique. Il nous a paru intéressant de déterminer si la composition observée est particulière à l'ocytocine des mammifères, ou au contraire se rencontre chez l'ocytocine d'espèces appartenant à d'autres classes de vertébrés.

Le dépouillement de 6,000 têtes de poulet* a fourni 4 g de poudre acétonique de post-hypophyses titrant environ 0.4 unité U.S.P. ocytocique ou vasopressique/mg. Deux préparations ont pu être effectuées, en utilisant chaque fois environ 2 g de matière première. Le procédé de purification employé est celui de ACHER, LIGHT ET DU VIGNEAUD[7] qui met en jeu l'adsorption de l'ocytocine et de la vasopressine sur une protéine post-hypophysaire, la neurophysine: Le premier stade est une précipitation spécifique du complexe à partir de l'extrait par le chlorure de sodium, le deuxième une élimination de la protéine-support par l'acide trichloracétique et le troisième une séparation chromatographique de l'ocytocine et de la vasopressine. Les essais préliminaires ont montré que la précipitation du complexe par le chlorure de sodium s'effectuait ici avec un mauvais rendement, soit que la neurophysine du poulet ait une solubilité particulière, soit qu'elle ait subi une dégradation, les glandes n'ayant pas été prélevées dès la mort des animaux. Cependant le procédé a pu être mis en oeuvre en effectuant un apport de neurophysine étrangère. En effet il est possible de reconstituer le complexe à partir de ses trois éléments, en utilisant des neurophysines d'origines variées[8].

2 g de poudre acétonique post-hypophysaire (940 unités ocytocique et 840 unités vasopressique) sont extraits à 5° deux fois par 60 ml H_2SO_4 0.01 *N*. Après élimination des substances insolubles, l'extrait (106 ml, pH 5.1) est ajusté à pH 3.9 avec de H_2SO_4 *N*, et on ajoute 168 mg de neurophysine de cheval. Après solubilisation de la protéine, on ajoute du NaCl de façon à avoir une teneur en sel de 10 %: le complexe précipite et on laisse à 5° pendant une nuit. Le précipité formé est séparé par centrifugation et redissous dans 18 ml d'acide acétique 0.03 %. On dialyse contre l'eau jusqu'à élimination totale des chlorures (rendement des deux activités 90–95 %). Le dialysé est acidifié avec de l'acide acétique (volume final 20 ml d'acide acétique à 0.25 %) et la neurophysine est précipitée au moyen de l'acide trichloracétique à 5 % dans les conditions antérieurement décrites[7]. Le liquide surnageant qui contient l'ocytocine et la vasopressine est débarrassé de l'acide trichloracétique par passage sur une colonne d'Amberlite-IR 45 (solution finale: 62 ml à pH 4 contenant 82 % de l'activité ocytocique et 74 % de l'activité vasopressique). La séparation de l'ocytocine et de la vasopressine est effectuée par chromatographie de gradient au moyen d'acétate d'ammonium sur une colonne d'Amberlite-IRC 50 dans les conditions déjà décrites[4–7]. La récupération de l'ocytocine est quantitative (770 unités). Les tubes contenant l'activité sont réunis et l'acétate d'ammonium est chassé par évaporation sous vide.

L'ocytocine du poulet se comporte comme l'ocytocine des mammifères au cours de la chromatographie sur colonne d'Amberlite-IRC 50 et au cours de la chromatographie sur papier dans les solvants *n*-butanol–acide acétique–eau (40:10:50) et

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* Nous sommes heureux de remercier ici la Société Industrielle de Transformation de Produits Agricoles (Auxonne) qui nous a très aimablement fourni les têtes de poulet.

n-butanol–acide acétique–eau–pyridine (30:6:24:20). Les comportements au cours de l'électrophorèse sur papier à pH 3.5, 6.5 et 8.6 sont également identiques. L'hydrolyse du produit fournit les acides aminés constituants de l'ocytocine des mammifères avec en plus de faibles quantités d'alanine, de sérine et d'arginine. Une purification plus poussée a été obtenue par chromatographie préparative sur papier: l'ocytocine du poulet (180 unités), purifiée sur Amberlite IRC-50 et débarrassée de l'acétate d'ammonium, est chromatographiée dans le solvant *n*-butanol–acide acétique–eau, l'ocytocine du cheval étant utilisée comme témoin. Après révélation de la bande témoin, on élue le matériel correspondant à l'ocytocine, et on hydrolyse par HCl 6 *N*, à 110° pendant 20 h. La composition en acides aminés du produit, établie par chromatographie semi-quantitative sur papier, est la suivante: $(Cys)_2$, Tyr,Ileu,Asp,-Glu,Pro,Leu,Gly, composition identique à celle de l'ocytocine des mammifères.

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Studies of DPNH oxidase: on the mechanism of reaction

Previously[1], we have shown that purified DPNH oxidase contains bound diphosphopyridine nucleotide in a fluorescent form, suggesting that it is bound to the enzyme in a reduced state. DPN containing [8-^{14}C]adenine was used to study the mode of binding of the pyridine nucleotide to DPNH oxidase and the relation of the binding to enzymic catalysis. Labeled DPN was obtained from Schwarz Laboratories, Mt. Vernon, New York. Concentrations of DPN and protein were determined as described previously[1]. [^{14}C]DPN was converted to [^{14}C]DPNH by reduction with $Na_2S_2O_4$, excess dithionite being removed by aeration. DPNH oxidase was incubated at room temperature with either [^{14}C]DPNH or [^{14}C]DPN for 20 min and was then washed repeatedly by centrifugation until the wash fluid was free of ^{14}C activity. The washed DPNH oxidase was analysed for total pyridine nucleotide and for ^{14}C-labeled pyridine nucleotide. Table I shows the exchange of [^{14}C]DPN and [^{14}C]-DPNH, at varying concentrations of labeled pyridine nucleotide, with the pyridine nucleotide originally bound to the DPNH oxidase. Only in Expt. 6, after incubation

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Abbreviations: DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide.

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with very high levels of [^{14}C]DPNH, was the final concentration of enzyme-bound pyridine nucleotide higher than that determined before addition of the labeled nucleotide; in all other instances the two values were equal demonstrating no net uptake of pyridine nucleotide. As shown in the Table, [^{14}C]DPN and [^{14}C]DPNH both exchange with the enzyme-bound pyridine nucleotide to the same degree, and the extent of the exchange is directly dependent on the amount of labeled pyridine nucleotide added. Subsequent assays with alcohol dehydrogenase showed that there was no disappearance of pyridine nucleotide during the course of these experiments since added DPNH was quantitatively recovered as DPN after its reaction with DPNH oxidase. It would therefore appear that the exchange reaction is not dependent on enzymic activity. The non-enzymic nature of the exchange is more directly shown by data from experiments similar to those presented in Table I but performed under anaerobic conditions and in the presence of cyanide. The results were identical to those presented in Table I.

The results presented demonstrate that the enzymic oxidation of DPNH by DPNH oxidase is not mediated by a direct exchange of substrate (DPNH) with the enzyme-bound pyridine nucleotide. Although there is an exchange of added pyridine nucleotide with that bound to the enzyme, this exchange is dependent on the concentration of pyridine nucleotide added and not on enzymic activity.

TABLE I

EXCHANGE OF ^{14}C-LABELED PYRIDINE NUCLEOTIDE WITH ENZYME-BOUND PYRIDINE NUCLEOTIDE

Expt.	*^{14}C-labeled pyridine nucleotide added**		*Exchange of labeled pyridine nucleotide with bound pyridine nucleotide***	
	[^{14}C]DPNH	*[^{14}C]DPN*	*[^{14}C]DPNH*	*[^{14}C]DPN*
1	3	3	0	0
2	6	6	12	12
3	20	20	22	22
4	190	190	87	87
5	215	—	91	—
6	432	—	100	—

* Expressed as a multiple of the enzyme-bound pyridine nucleotide.

** Expressed as the per cent of enzyme-bound pyridine nucleotide which exchanged with the added ^{14}C-labeled pyridine nucleotide.

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Phosphorylation coupled to α-ketoglutarate oxidation in Ehrlich-ascites-cell mitochondria

Ehrlich-ascites-cell mitochondria, like those of other tumours, exhibit a pronounced DPN requirement for maximal oxygen uptake[1,2]. However, little information is available concerning the phosphorylations coupled to oxidation of substrates of the tricarboxylic-acid cycle in these mitochondria. KUN, TALALAY AND WILLIAMS-ASHMAN[3] reported P/O ratios for Ehrlich mitochondria respiring with succinate in a medium without DPN or the DPNase inhibitor, nicotinamide. LINDBERG, LJUNGGREN, ERNSTER AND RÊVÉSZ[4] quote P/O ratios for pyruvate, citrate, α-ketoglutarate, succinate and malate in a medium again without DPN or nicotinamide. We have measured P/O ratios for succinate, ascorbate, α-ketoglutarate and L-glutamate in similarly unfortified media, and have reported preliminary observations concerning the influence of DPN and nicotinamide on α-ketoglutarate and L-glutamate oxidation[2]. This communication describes a further examination of the effect of DPN and nicotinamide additions on the phosphorylations associated with α-ketoglutarate oxidation.

Mitochondria were isolated in 0.25 *M* sucrose according to the method of HAWTREY AND SILK[2] and were allowed to stand at 0° for periods of 4–5.5 h, in order to deplete them of pyridine nucleotides under mild conditions and permit examination of the effect of adding back DPN. Such ageing of tumour mitochondria is reported by KIELLY[5] to result in a lowered capacity for phosphorylation which can be largely restored by subsequent addition of DPN. Mitochondria of normal cells do not, however, appear to undergo loss of phosphorylation activity on corresponding ageing.

TABLE I

EFFECT OF DPN AND NICOTINAMIDE ADDITIONS ON PHOSPHORYLATION ASSOCIATED WITH α-KETOGLUTARATE OXIDATION IN AGED EHRLICH-ASCITES-CELL MITOCHONDRIA

Flasks contained the following basic medium in a final vol. of 3.0 ml: – K, Na phosphate buffer (pH 7.4), 0.045 *M*; D-glucose, 0.034 *M*; KF, 0.015 *M*; $MgCl_2$, 0.008 *M*; ADP, 0.0025 *M*; AMP, 0.001 *M*; ethylenediaminetetra-acetic acid (pH 7.4), 0.002 *M*; KCl, 0.01 *M*; sucrose, 0.062 *M*; α-ketoglutarate, 0.0066 *M*; malonate, 0.02 *M*; cytochrome *c*, $9.8 \cdot 10^{-6}$ *M*; hexokinase (crystalline), 1–2 mg; mitochondrial suspension (aged 4–5.5 h in sucrose at 0°) in 0.25 *M* sucrose (0.4 ml) equivalent to 2.11 ± 0.29 mg protein determined according to CLELAND AND SLATER[6]. Temp. 30°. The reaction was stopped after 30–40 min by addition of 1.0 ml 30 % trichloroacetic acid. Phosphate uptake was determined according to FISKE AND SUBBAROW[7]. The values given are the means ± the average deviations of results obtained in a total of 11 determinations with different batches of mitochondria.

Special additions	*O_2 consumed µatoms/mg protein/h*	*P esterified µatoms/mg protein/h*	*P/O*
DPN ($2.4 \cdot 10^{-3}$ *M*) + nicotinamide (0.02 *M*)	5.40 ± 1.02	11.60 ± 2.78	2.17 ± 0.35
DPN ($2.4 \cdot 10^{-3}$ *M*)	5.51 ± 1.10	8.35 ± 3.14	1.49 ± 0.40
Nicotinamide (0.02 *M*)	3.43 ± 1.00	7.28 ± 2.07	2.17 ± 0.49
None	3.77 ± 1.10	6.45 ± 4.10	1.63 ± 0.92

In accord with previous results[2] and the findings of other workers concerning tumour mitochondria[1,5], we have shown that addition of DPN causes a significant increase in the oxygen uptake of aged Ehrlich mitochondria respiring with α-keto-

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Abbreviations: DPN, DPNH, oxidised and reduced diphosphopyridine nucleotide; AMP, ADP, adenosine mono- and di-phosphate.

glutarate as substrate (Table I). Oxygen uptake was increased approximately 46 % by addition of $2.4 \cdot 10^{-3}$ *M* DPN to the medium. Nicotinamide (0.02 *M*), on the other hand, had no significant effect on the oxygen uptake in either DPN-fortified or unfortified media.

The phosphorylation activity of mitochondria aged 4–5.5 h was markedly influenced by addition of nicotinamide which raised the P/O ratio by approximately 46 % in media fortified with DPN and by approximately 33 % in media without DPN. The effect was greatest with low concentrations of mitochondrial protein.

TABLE II

OXIDATION OF DPNH BY AGED EHRLICH-ASCITES-CELL MITOCHONDRIA

Flasks contained the basic medium of Table I without malonate. Special additions as indicated. Temp. 30°. Inorganic phosphate was liberated instead of esterified.

Special additions	*Mitochondrial protein mg*	*O_2 consumed µatoms/mg protein/h*	*P esterified µatoms/mg protein/h*
DPN ($2.4 \cdot 10^{-3}$ *M*) + alcohol dehydrogenase (0.4 mg) + ethanol (0.04 *M*)	1.44	4.49	+ 2.40
DPN ($2.4 \cdot 10^{-3}$ *M*) + alcohol dehydrogenase (0.4 mg) + ethanol (0.04 *M*) + nicotinamide (0.02 *M*)	1.44	4.71	+ 1.42

In a medium without nicotinamide, DPN fortification seems to cause a lowering of approximately 9 % in P/O ratio. Although DPN addition raised the oxygen uptake, the esterification of inorganic phosphate was not correspondingly increased, with the result that lower P/O ratios were observed in the presence of DPN (Table I). Similar results were obtained by KIELLEY[5] confirming that externally added DPN does not completely restore the phosphorylation activity lost on ageing.

Mitochondria aged only 1 h showed a P/O ratio of approximately 1.56 (average of 1.49, 1.64) in the absence of DPN and nicotinamide, while mitochondria aged for 4–5.5 h show a P/O ratio of approximately 1.63 in the same medium (Table I).

Externally added DPNH is readily oxidised by aged Ehrlich mitochondria but the oxidation does not appear to be coupled to phosphorylation. In fact a small output of inorganic phosphate was observed in experiments in which the DPNH was generated *in situ* by addition of DPN, ethanol and alcohol dehydrogenase to the medium (Table II).

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On the mechanism of sulfide oxidation in biological systems

Baxter and Van Reen[1] recently observed that when inorganic sulfide was oxidized in the presence of rat-liver preparations, determinations of thiosulfate by two different methods sometimes gave different results. The methods employed were a conventional iodometric titration and an enzymic method[2], based on the conversion of thiosulfate to thiocyanate in the presence of cyanide and the enzyme rhodanese. The rhodanese method always gave higher values than the iodometric titration except when the sulfide had been completely oxidized, in which case essential agreement between the two methods was observed. This suggested that sulfide was oxidized to thiosulfate through an intermediate, which behaved as thiosulfate in the rhodanese assay system but did not reduce iodine. An important difference between the two methods for thiosulfate assay was that the iodometric titrations were carried out on deproteinized samples whereas the enzymic assays were carried out on the untreated reaction system. If, however, deproteinized samples were also used for the enzymic determinations essential agreement between the two methods was again obtained. This was taken as evidence for the intermediate being protein bound, and it was further suggested that this intermediate was formed through sulfitolysis of a protein disulfide bond according to $RSSR + HSO^-_3 \rightarrow RSH + RSSO^-_3$. (It was assumed that sulfide was oxidized to sulfite in a preceding reaction.) $RSSO^-_3$ was suggested to be the unknown intermediate and was classified as a thiosulfonate. Thiosulfonates are, however, of the formula RSO_2S^-, whereas compounds of the formula $RSSO_3^-$ should be classified as thiosulfate esters[4,5]. The latter are not converted to thiocyanate by cyanide and rhodanese[3], and the reaction mechanism suggested by Baxter and Van Reen is consequently untenable. We have now been able to identify the intermediate described by Baxter and Van Reen as follows.

It was previously reported[6] that certain heme compounds catalyze the oxidation of inorganic sulfide to thiosulfate, and we observed that a compound similar in behaviour to the intermediate of Baxter and Van Reen was formed also in these reactions. Thus when inorganic sulfide (0.005 *M*) and hemin (5.4 μg/ml) in 0.133 *M* phosphate buffer, pH 7.2, was shaken in air, it was found that after 30 min the system contained 0.23 μmole S_2O_3/ml as determined by iodometric titration whereas the rhodanese method gave 1.13 μmoles S_2O_3/ml. Obviously a protein-bound intermediate could not have been formed in this case as no protein was present in the system. It has previously been demonstrated that when sulfide is oxidized in the presence of liver extracts[7] or heme compounds[6] thiosulfate is not the only reaction product as free sulfur is also formed. The possibility was first considered that sulfur was the unknown intermediate, although Baxter *et al.*[2] stated that sulfur did not interfere in the rhodanese assay for thiosulfate. Sulfur, if present in a colloidal form[3,8], is actually converted to thiocyanate in the presence of cyanide and liver extracts and we observed in fact that colloidal sulfur interfered to a slight but significant extent in the rhodanese assay for thiosulfate, if the latter was carried out on a liver homogenate. But the unknown intermediate could not be identical with colloidal sulfur, as the presence of rhodanese was found to be necessary for the reaction between the intermediate and cyanide, whereas rhodanese does not catalyze the reaction between sulfur and cyanide[3]. However, when sulfide is incompletely oxidized to sulfur, the latter will react with remaining sulfide to give a polysulfide[*]. It was now observed that inorganic polysulfide behaved as the unknown intermediate in the two methods for thiosulfate assay. Thus when 3 ml of a polysulfide solution (containing 40 µmoles Na_2S and 10 µmoles S per ml) were added to 27 ml of 0.133 *M* phosphate buffer, pH 7.2, and the thiosulfate content immediately determined, the iodometric method gave 0.22 µmole S_2O_3/ml[**], whereas the rhodanese method gave 1.01 µmoles "apparent" S_2O_3/ml. It was also demonstrated that rhodanese was necessary for the conversion of the polysulfide to thiocyanate. From these observations it was concluded that the unknown intermediate in sulfide oxidation was identical with inorganic polysulfide. The observation of BAXTER AND VAN REEN[1] that deproteinization (with $ZnCO_3$) abolished the interference given by the intermediate in the rhodanese assay is explained by the fact that polysulfide is decomposed and removed from the system by this treatment.

It was previously[3] reported that the spontaneous reaction between a polysulfide (with a ratio of sulfide to sulfur corresponding to a disulfide) was so rapid even at a neutral pH that any catalytic effect of rhodanese could not be demonstrated. These experiments were, however, made at a "disulfide" concentration of 0.05 *M*, but if this was lowered to 0.001 *M* the spontaneous reaction became so slow that it was possible to demonstrate that "disulfide" under these conditions was a good sulfur donor for rhodanese (Table I).

TABLE I

INORGANIC "DISULFIDE" AS SUBSTRATE FOR RHODANESE

The test system of pH 7.4 contained 1 µmole "disulfide" (Na_2S_2) or thiosulfate as indicated, 50 µmoles cyanide, 60 µmoles phosphate, 50 µg bovine serum albumin and 7.8 µg crystalline rhodanese (the latter was omitted from the "blank" system) in a final volume of 1.0 ml. Reaction time, 5 min at 20°. The thiocyanate formed was determined colorimetrically[3].

System	*Sulfur donor*	*CNS^- formed µmoles*
Blank	Thiosulfate	0.02
Rhodanese	Thiosulfate	0.44
Blank	"Disulfide"	0.16
Rhodanese	"Disulfide"	0.78

The oxidation of polysulfide ("disulfide") to thiosulfate was also studied and found to be considerably more rapid than that of sulfide in the absence of any added catalyst. Free hemin and hemoglobin had no significant catalytic action on polysulfide (Table II). As these heme compounds catalyze the oxidation of sulfide to thiosulfate[6], their catalytic activity must in this case be due to the fact that they catalyze the oxidation of sulfide to polysulfide, but the oxidation of the latter then proceeds spontaneously. Liver extracts, on the other hand, also catalyzed the oxidation of "disulfide" to thiosulfate (Table II) and consequently the "sulfide oxidase" in liver cannot be identical with hemoglobin, as we previously suggested[6]. The existence of a non-hemoglobin sulfide oxidase in liver has previously been advocated by BAXTER *et al.*[2].

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[*] For a discussion on the chemistry of inorganic polysulfide solutions, see ref.[9]. They actually contain equilibrium mixtures of higher and lower polysulfides.

[**] Formed by rapid autoxidation of the polysulfide.

TABLE II

THIOSULFATE FORMATION FROM SULFIDE AND "DISULFIDE"

The test system of pH 7.2 contained 15 µmoles Na_2S or 7.5 µmoles Na_2S_2, 400 µmoles phosphate and catalyst as indicated in a final volume of 3 ml. Incubation was carried out with shaking in Warburg vessels for 15 min at 37° and thiosulfate then determined colorimetrically[4]. The values obtained were corrected for corresponding "blanks" (0.10–0.25 µmole $S_2O_3^{2-}$ in case of sulfide and 1.1–1.5 µmoles $S_2O_3^{2-}$ in case of "disulfide").

Catalyst	*Substrate*	*$S_2O_3^{2-}$ formed µmoles*
Hemin (32.5 µg)	Sulfide	1.37
Hemoglobin (10 mg)	Sulfide	1.85
Liver extract*	Sulfide	1.50
Hemin (32.5 µg)	"Disulfide"	0.10
Hemoglobin (10 mg)	"Disulfide"	0.13
Liver extract*	"Disulfide"	1.43

* Prepared according to BAXTER *et al.*[2]. Each vessel contained 0.34 g liver (fresh weight).

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Crystalline synthetic porphyrin c

Synthetic porphyrin *c* was prepared more than twenty years ago by THEORELL[1] who heated protoporphyrin with L-cysteine in mineral acid solution. ZEILE AND MEYER[2], on the other hand, heated protoporphyrin with HBr and subsequently fused the crude adduct with L-cysteine. Both procedures gave non-crystalline products with the correct analytical data and the expected properties for the structure given in Fig. 1.

In this laboratory, during the course of preparation of porphyrin *c* to be used for metal-ion complexing studies, two modifications in the method of ZEILE AND MEYER[2] were introduced which led ultimately to a crystalline product. Thus in the present instance (a) the porphyrin–HBr adduct was fused with L-cysteine in an oil bath under controlled conditions instead of over an open flame, and (b) the crude product obtained after fusion was subjected to partition chromatography with butanol–acetic acid–0.02 *N* HCl on a Celite column.

1 g crystalline protohemin was sealed in a glass tube with 20 ml 40 % HBr in acetic acid. After heating for 1.5 days at 37° the tube was opened and the contents poured out into a crystallizing dish. The solvent and excess HBr were removed under slightly reduced pressure in the presence of NaOH in a desiccator. Several additions of 10-ml volumes of glacial acetic acid were introduced over a period of 2–3 days in order to remove the last traces of HBr.

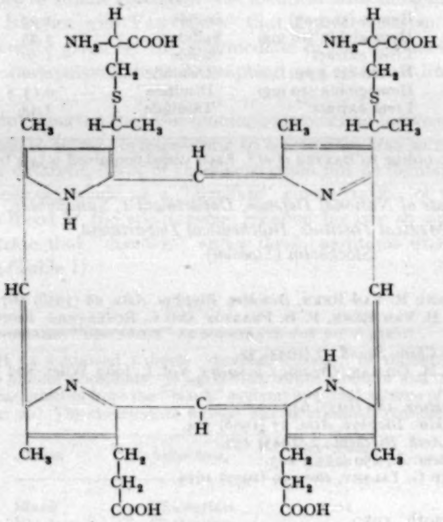

Fig. 1. Structure of porphyrin *c*.

The tacky material was thoroughly stirred with 3 g L-cysteine·HCl and the mixture stored under full vacuum overnight. A dry, crumbly product was obtained.

An oil bath was heated to 160° and the sample, contained in a beaker suspended by a wire handle, was introduced. The mixture, which melted almost instantly, was stirred for 3 min and then allowed to cool to room temperature. The crude product was dissolved in water and dil. NaOH then added to give a pH of 4. The precipitated porphyrin was collected by centrifugation and dried in a desiccator.

A 3.2 × 20 cm partition column of purified Celite with butanol–acetic acid–0.02 *N* HCl (4 : 1 : 5) as solvent was prepared as described by TUPPY AND PALÉUS[8]. A 200-mg sample of the crude product was dissolved in the minimum quantity of external solvent and applied to the surface of the column. Three principal bands were found. The fast and intermediate-rate bands were of about equal intensity; the slowest flowing band was a minor constituent. The intermediate-rate band only was collected and the porphyrin extracted from the organic layer with three 12-ml portions of 1 *N* HCl. The combined extracts were adjusted to pH 4 with 6 *N* NaOH and set aside in the dark for crystallization. After standing overnight the rosettes were filtered, washed with a small amount of cold water and dried in a desiccator. The yield was about 100 mg, *i.e.* about 50% of the total material applied to the column. Additional 200-mg batches of crude porphyrin were purified by passage through the same column.

The rosettes did not melt below 300°. The isolated compound was highly fluorescent and gave a positive ninhydrin reaction. The solubility, titration and spectral data were, in general, in good agreement with those reported by THEORELL[4] for the natural product. The titration curve closely resembled that shown by THEORELL[4] (Fig. 2 p. 244), *i.e.* the mid-points of the first and second buffer zones were at pH 5.6 and 9.2, respectively. The neutral equivalent calculated at pH 7.2 was 806, a figure in excellent agreement with the theoretical value of 804. The shape of the light-absorption curve in 1 *N* HCl was the same as that reported by THEORELL[4] (Fig. 1, p. 243) and the extinction coefficient of $1.65 \cdot 10^4$ at 553 mμ was confirmed. The Soret maximum in 1 *N* HCl was at 406 mμ and gave an extinction coefficient of $3.12 \cdot 10^5$ in contrast to the value $2.48 \cdot 10^5$ reported for natural porphyrin *c*. A single, elongated spot was observed on paper chromatograms developed with a lutidine–water (1:1) solvent. The R_F ratio for porphyrin *c*/protohemin was 1.0.

A number of different samples dissolved at varying concentrations in very dilute HCl did not transmit sufficient light to permit an exact determination of the specific rotation. The substance can be assumed to be optically active since natural cysteine was employed and racemization would not have been anticipated during the synthesis. Neither the method of preparation nor the available characterization data will allow an entirely unequivocal choice between an α or a β attachment of the sulfur atom to the porphyrin side chain. However, it has been found that the C–S lyase[5] of *Albizzia lophanta* splits synthetic porphyrin *c* either at a negligible rate or not at all. Although this enzyme cleaves a number of S-alkyl cysteines, the branched-chain derivatives appear to be acted upon much less readily than the corresponding straight-chain S-alkyl compounds[6]. The failure of the C–S lyase to attack our preparation would therefore favor the structure shown, *i.e.*, the α-isomer.

Analyses

Found: N, 10.71, 10.62; S, 7.94. Calc. for $C_{40}H_{48}N_6S_2O_8$: N, 10.44; S, 7.97.

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S-Adenosylmethionine formation by barley extracts[*]

The finding that (—)-S-adenosyl-L-methionine can function as a methyl donor in the enzymic formation of N-methyltyramine, hordenine, and gramine, while L-methionine, L-methionine sulfoxide, and S-methyl-L-methionine are inactive in the same plant-enzyme systems[1] strongly suggests that AMe is an intermediate on the natural pathway of transmethylation in these plants. Since the presence of AMe has not been reported in higher plants, it was important to demonstrate that this compound may indeed be formed by plants. The present paper reports such a demonstration.

L-[*Me*-^{14}C]methionine was incubated with extracts of barley seedlings in the presence of $MgCl_2$, KCl, adenosine-5′-triphosphate and a small pool of AMe. As is shown in Table I, the AMe fraction contained radioactivity only after incubation with radioactive methionine and enzyme. L-methionine sulfoxide did not replace methionine.

TABLE I

FORMATION OF S-ADENOSYLMETHIONINE BY BARLEY EXTRACTS

Substrate	*Counts/min in AMe fraction*	
	Minus enzyme	*Plus enzyme*
L-[*Me*-^{14}C]methionine	22	480
L-[*Me*-^{14}C]methionine sulfoxide	23	32

Each vessel contained in μmoles: tris(hydroxymethyl)aminomethane, pH 7.6, 100; KCl, 100; $MgCl_2$, 50; adenosine-5′-triphosphate, 13.7; (—)-S-adenosyl-L-methionine[2], 1.0; either L-[*Me*-^{14}C]-methionine, 0.11 ($103 \cdot 10^3$ counts/min) or L-[*Me*-^{14}C]methionine sulfoxide (synthesized by treatment of L-[*Me*-^{14}C]methionine with H_2O_2), 0.2 ($127 \cdot 10^3$ counts/min). Vol., 0.50 ml. The enzyme was prepared by grinding 6-day-old barley seedlings with sand and 1.5 vol. (w/v) potassium phosphate buffer, pH 7.0, 0.02 *M*. The suspension was filtered through a plug of glass wool and 1.0 ml of the filtrate added to the reaction mixtures as indicated. Incubation was at 24° for 6 h. After incubation, 0.18 ml 30% $HClO_4$ was added to all vessels and enzyme to "minus enzyme" vessels. 7.5 μmoles carrier L-methionine or methionine sulfoxide were then added. Aliquots of the deproteinized supernatant fluid were neutralized and chromatographed on columns of Amberlite IRC-50 (XE-64), 6 × 1 cm, buffered at pH 7.0[3]. Aliquots of the fractions containing AMe were counted in a thin-window, gas-flow counter.

The AMe fraction was lyophilized and chromatographed again on IRC-50 (XE-64) buffered at pH 7.6 with ammonium phosphate. Under these conditions, AMe is eluted with dilute pH 7.6 buffer after a lag and over a range of several column volumes. It was found that the radioactivity was eluted in a manner parallel to the AMe as judged by its u.v. absorption.

The AMe fractions were combined, passed over a column of Dowex-1 formate and lyophilized. The residue was dissolved in water, the solution brought to pH 5 by the addition of formic acid and heated at 100° for 20 min to decompose the AMe to 5′-methylthioadenosine and homoserine (or homoserine lactone)[4,5]. A paper chromatograph of a concentrate of this solution showed that essentially all the radioactivity was present in the 5′-methylthioadenosine.

A similar series of experiments was carried out using L-[2-^{14}C]methionine.

Abbreviation: AMe, (–)-S-adenosyl-L-methionine.

[*] Paper no. 20 in a series on enzymic mechanisms in transmethylation.

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Again radioactivity was incorporated into AMe as judged by its behavior on the two ion-exchange columns. Decomposition was performed as above, the resulting radioactive material was desalted by adsorption on Dowex-1 (OH^-) followed by elution with HCl[6] and was identified by paper chromatography as homoserine. In a third set of experiments, L-[1-^{14}C]methionine was shown also to be incorporated into AMe which on decomposition gave rise to radioactive homoserine.

To confirm and extend these findings, some of the [*Me*-^{14}C]AMe was subjected to treatment with a partially purified yeast enzyme which cleaves AMe to 5′-methylthioadenosine and to α-amino-γ-butyrolactone[3]. The reaction was allowed to proceed until 90% of the AMe was decomposed. The specific activities of the initial AMe and the 5′-methylthioadenosine which was formed were the same within experimental error (15,100 and 16,000 counts/min/μmole respectively). Because of the known substrate specificity of this enzyme, these results confirm not only that the radioactivity was in S-adenosylmethionine[3] but clearly establish the configuration at the asymmetric sulfur and α-amino-carbon atoms of the AMe as—and L[3,7].

The facts that AMe is formed by barley and that it is used by this plant and by millet in a group of alkaloid-forming transmethylations suggests that this compound is the predominant methyl donor in plants as it is known to be in animals and microorganisms.

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Inhibition of yeast carboxylase by acetaldehyde

It has been known for some time, in fact since the description of carboxylase by NEUBERG[1], that the velocity of the reaction

$$\text{pyruvate} \longrightarrow \text{acetaldehyde} + CO_2$$

catalyzed by yeast carboxylase quickly decreases even in the presence of an excess of pyruvate[1,7]. The deviation of the curve of the CO_2 evolution from the straight line to be expected at zero-order conditions has generally been attributed to inhibition of the enzyme by the acetaldehyde formed[1–5], especially as the enzyme is relatively stable during incubation without substrate[2,3,5]. There are, however, to our knowledge no published data deciding the type of inhibition or the question whether the observed decrease is only due to acetaldehyde inhibition.

In order to decide the type of inhibition, the time curve of CO_2 evolution from pyruvate by fresh baker's yeast, which had been made permeable to pyruvate by repeated freezing and thawing, was obtained by the conventional Warburg technique. The reaction velocity at different times was obtained by drawing slopes to the curves at different points or by measuring the CO_2 production at relatively short intervals. The acetaldehyde concentration at any point was assumed to be equal to the amount of CO_2 evolved divided by the volume of the incubation mixture. The amount of pyruvate added was saturating.

In the case of apparent non-competitive or un-competitive inhibition, assuming reversible combination of the enzyme with n molecules of acetaldehyde according to $E + nA \rightleftharpoons EA_n$, the velocity v at any time or acetaldehyde concentration should be proportional to the concentration of uninhibited enzyme at saturating levels of pyruvate, viz. $v = k(e - ea_n)$. This yields

$$v = \frac{\mathrm{d}a}{\mathrm{d}t} = \frac{v_0}{1 + \dfrac{a^n}{K_n}} \tag{I}$$

where a is the acetaldehyde concentration, e the total concentration of enzyme ($e \ll a$), K_a the dissociation constant of the acetaldehyde–carboxylase complex, and v_0 ($= ke$) the velocity at $a = 0$. A plot of $1/v$ against a^n should yield a straight line. Fig. 1 shows that this is the case for $n = 1$ and also indicates the correctness of the type of inhibition assumed. From the extrapolated curve in Fig. 1 and other similar curves K_a could be obtained. In 0.1 M acetate buffer of pH 5.1, K_a is about $4 \cdot 10^{-3}\,M$.

As the calculation of the velocity involved the drawing of slopes to the product-time curve and K_a was obtained by extrapolation a control of the procedure by a

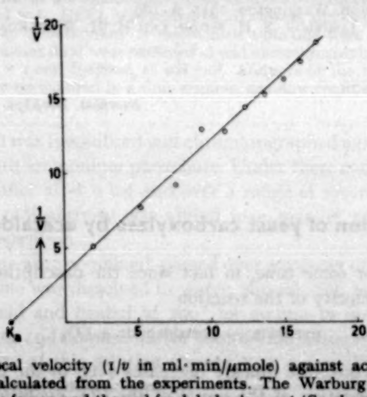

Fig. 1. Plot of reciprocal velocity ($1/v$ in ml·min/μmole) against acetaldehyde concentration (a in μmoles/ml) as calculated from the experiments. The Warburg vessels contained 1.5 ml suspension of four-times frozen and thawed fresh baker's yeast (*Saccharomyces cerevisiae*, Koningsgist) in 0.1 M Na acetate buffer, pH 5.1 (19.2 mg fresh yeast/ml final suspension) and 0.3 ml 3.5% sodium pyruvate (final concn., 53 μmole/ml) in acetate buffer. All points are the mean of triplicate determinations. The drawn curve was obtained by the method of least squares. A K_a value was obtained of $3.7 \cdot 10^{-3}\,M$ at 27.0°.

more direct use of the experimental data was considered desirable. Integration of the relation in eqn. (I) yields

$$v_0 \cdot t = a + \frac{a^2}{2K_a} \tag{II}$$

The right-hand term of this equation divided by t should therefore give a constant value, viz. the initial velocity v_0. Table I shows that this relation holds for the experiment of Fig. 1 which is typical of all experiments done. This relation affords a good method of measuring carboxylase activity, if K_a has first been determined for the experimental conditions used.

TABLE I

Experimental conditions as described in legend to Fig. 1 (for explanation see text).

t (min)	a = acetaldehyde concentration at t (µmoles/ml)	$\frac{a + \frac{a^2}{2K_a}}{t} = v_0$
10	1.98	0.250*
30	5.25	0.296
50	7.61	0.304
70	9.46	0.303
90	11.01	0.299
110	12.49	0.300
130	13.78	0.298
150	15.09	0.300
170	16.28	0.301
190	17.36	0.300

* The value after 10 min in all experiments appeared to be lower owing to insufficient penetration of substrate, and/or incomplete gas equilibration.

It is thus clear that the carboxylase in fresh baker's yeast is inhibited in an apparent non-competitive or uncompetitive manner by the acetaldehyde formed at saturating pyruvate concentrations, with an inhibitor dissociation constant of about $4 \cdot 10^{-3}$ M in 0.1 M acetate buffer, pH 5.1. Further experiments in our laboratory have shown that this constant is pH and/or buffer dependent in the range of carboxylase activity. With air-dried brewer's yeast similar curves were obtained confirming the type of inhibition assumed.

We also tried to obtain the same curves by adding different amounts of acetaldehyde to our incubation mixture. Essentially the same results were obtained, but the added acetaldehyde appeared to be only about 90% as effective as the same amount formed by the yeast.

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Effect of diet on the occurrence of S-methylcysteine and the free amino acid pattern in insect blood

The remarkably high concentration of free amino acids found in the blood or haemolymph of various insects constitutes a biochemical characteristic of the Class[1-3]. Although this fact has been known for over 30 years[4], neither the origin nor the significance of this high titer has yet been explained. During the course of studies on the free amino acids of various biological fluids and tissue extracts we have re-examined the larval haemolymph of the Southern Armyworm, *Prodenia eridania*. In thus extending the earlier qualitative data of PRATT[5] we have demonstrated for the first time the occurrence of free S-methylcysteine in animal as opposed to plant[6] tissue. The experiments here reported also show that the occurrence of this amino acid is dependent upon the presence of a precursor in the diet.

Pooled haemolymph from 30–40 fully grown larvae raised entirely on kale (*Brassica oleracea*) was deproteinized with absolute ethanol to a final concentration of 80% (v/v). The precipitate obtained after centrifugation was washed in the centrifuge with 80% ethanol, and the combined supernatant and washings desalted by passage through a 1 × 4 cm column of Dowex 50, 50–100 mesh, in the H^+ form. The column was washed with deionized water until the pH of the effluent attained neutrality, following which the amino acids were eluted with 9 N NH_3. The excess NH_3 was removed *in vacuo* at 25° and the extract lyophilized. The residue was dissolved in a known volume of water, and suitable aliquots taken for determination of amino acid nitrogen[7] and for two-dimensional paper chromatography[8]. After spraying with ninhydrin an approximate quantitation of the amino acids was obtained by scanning each "spot" with a densitometer, employing the "maximum density" method[10]. The sum of the densities of the individual amino acid "spots" was equated to the total amino nitrogen applied to the paper, and the amount of each amino acid was then expressed as per cent of the total. Proline, which gives an atyptical ninhydrin color, was estimated by visual comparison with the most closely approximating ninhydrin "spot" of similar size.

A ninhydrin-reactive "spot" whose position on the chromatograms corresponded to that of free S-methylcysteine was conspicuous in the haemolymph of *P. eridania* larvae raised on kale. The material was characterized by co-chromatography with the known compound in two different solvents, and by its reaction with platinic iodide[10].

In order to determine whether the S-methylcysteine was formed *de novo* or was derived from the diet, 80%-ethanol extracts of kale were examined by procedures similar to those employed for the insect haemolymph. In addition, a batch of *P. eridania* was raised first on kale until about the second instar, by which stage the larvae were large enough to be transferred to, and to continue their development on potato slices. Haemolymph from these potato-fed larvae, and 80%-ethanol extracts of the potatoes were also exmined[11]. The data are presented in Table I.

The kale extracts did not contain any trace of S-methylcysteine, although its oxidation product S-methylcysteine sulfoxide[12,13] was present in high concentration. Since under our experimental conditions the sulfoxide was not artifically reduced to S-methylcysteine, it appears likely that the reduction is performed enzymically by the insect. Neither of the above S-containing amino acids was found in either the

TABLE I

FREE AMINO ACIDS IN *P. eridania* HAEMOLYMPH AND EXTRACTS OF FOOD PLANTS

A (—) indicates insufficient material to be detected on the chromatogram. Values in per cent of total.

Amino acid	P. eridania *raised on kale 1.45 mg amino N/ml haemolymph*	*Kale extract 0.891 mg amino N/g kale*	P. eridania *raised on potato 1.45 mg amino N/ml haemolymph*	*Potato extract 0.015 mg amino N/g potato*
Cystine and/or peptides	1.0	2.2	1.1	0.4
Histidine	8.7	0.4	5.7	1.8
Asparagine	0.7	2.1	2.5	4.4
Ornithine?	3.9	—	2.5	—
"Before lysine" unknown	—	1.0	—	—
Lysine	15.2	3.9	9.0	6.5
Arginine	0.8	2.8	3.2	7.2
"Below arginine" unknown	—	5.6	—	—
Aspartic acid	Trace	2.4	1.9	8.5
Glutamic acid	1.8	12.9	4.1	8.5
Glycine	6.7	0.9	3.7	1.4
S-methyl cysteine	2.3	—	—	—
S-methyl cysteine sulfoxide	1.4	12.5	—	—
Glutamine	8.1	15.1	12.8	16.6
Serine	11.1	7.3	12.8	4.1
Threonine	6.6	2.8	4.2	2.4
β-alanine	2.1	0.9	2.9	0.6
γ-aminobutyric acid	1.0	3.4	7.1	15.7
Alanine	4.6	5.4	6.5	1.8
Ethanolamine	—	3.7	—	0.9
Proline	10.0	4.0	6.4	1.8
α-aminobutyric acid	0.4	—	0.4	0.4
Valine	4.6	6.5	5.3	7.4
Methionine	—	—	1.9	1.8
Isoleucine	2.0	2.6	1.9	2.2
Leucine	2.4	0.5	1.3	1.4
Phenylalanine	1.3	0.3	1.1	1.3
Tryptophan	—	—	—	0.3
Tyrosine	2.6	—	1.9	1.4
Pipecolic acid	Trace	0.3	Trace	1.1

potato extracts or in the haemolymph of *P. eridania* raised on potatoes. We conclude, therefore, that the insect S-methylcysteine is derived from the corresponding sulfoxide contained in the diet.

Inspection of the Table reveals that although the proportions of individual free amino acids in the two types of haemolymph or in the two plant extracts may differ more or less widely, nevertheless a certain regularity exists. Thus by comparing the haemolymph amino acids with those of the corresponding diet on which the insects were raised, it appears that the insects can selectively accumulate particular amino acids (*e.g.* histidine, lysine, serine, proline, etc.) irrespective of their actual concentration in the diet. Conversely, the relative proportions of other haemolymph amino acids (*e.g.* asparagine, aspartic acid, arginine, etc.) are to a varying degree lower than that of the diet. This applies especially to those amino acids which are not normally constituents of proteins, viz. γ-aminobutyric acid, pipecolic acid, ethanolamine and the two unknown ninhydrin-positive "spots" from kale. This suggests that these latter amino acids are either metabolized and/or excreted or, in the extreme case where they are actually absent from the haemolymph, not absorbed. Their presence or absence as compared with the diet is thus an indication of the extent to which the insect controls its precise pattern of haemolymph-free amino acids.

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A simplified procedure for the preparation of phosvitin and vitellin*

The two major phosphoproteins of egg yolk are phosvitin and vitellin, the latter being in the form of a lipoprotein complex[1]. MECHAM AND OLCOTT[2] described a procedure for the isolation of phosvitin, containing 10 % phosphorus and accounting for nearly 70 % of total yolk phosphorus. The lipovitellin, first isolated by ALDERTON AND FEVOLD[3], was subjected to a detailed electrophoretic and ultracentrifugal study[4, 5] which revealed that this protein is a mixture of several components, involving the total phosvitin of egg yolk and the lipid-bound vitellin. The lipovitellin fraction of ALDERTON AND FEVOLD[3] thus promised to be good starting material for the preparation of both phosvitin and vitellin.

Butanol, which is being widely used to liberate the proteins bound to lipids[6], was employed in the present investigation to disrupt the lipid–protein complex of lipovitellin. This procedure, while rendering vitellin insoluble, incidentally released all the phosvitin into solution from which it could be recovered by isoelectric precipitation. The method adopted was as follows:

Yolks from 50 eggs were freed of adhering white and chalazae, and diluted with two volumes of distilled water. The emulsion was passed through a Sharples centrifuge[3] and the residue was taken up in 500 ml 10 % NaCl. Lipovitellin was precipitated by diluting the salt solution with 7–8 l water. The precipitate was collected by centrifuging, and dissolved in 500 ml 10 % NaCl. The saline extract was treated with 250 ml *n*-butanol and the solution was stirred at room temperature for 1 h after which it was kept in the cold for 24 h. After centrifugation, the top butanol layer which was orange in colour was siphoned off carefully. The bottom aqueous layer and the middle layer of thick precipitate were separated by filtration on a Buchner funnel. The precipitate was suspended in 500 ml 10 % NaCl, 250 ml *n*-butanol again added and the process was repeated. The aqueous layers were pooled. The insoluble vitellin was washed with water thrice to free it from NaCl, then repeatedly with acetone till the washings were colourless, finally with ether and then air-dried. The yield was about 20 g.

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For the preparation of phosvitin, the combined aqueous extract was shaken twice with 150-ml portions of ether in a separatory funnel, and the ether extracts discarded. The clear solution which was light yellow in colour was acidified with 2 *N* HCl with stirring to pH 1.8 and kept in the cold overnight to complete the precipitation. The gelatinous precipitate was centrifuged off, dissolved in 200 ml water by the addition of 2 *N* ammonia to pH 6.0, and then filtered through a fluted filter paper. The protein was reprecipitated by acidification to pH 1.8, and dissolved as before. The solution was treated with $MgSO_4$ to 0.4 *M* saturation and stirred for 1 h at room temperature. Enough water was added to bring down the concentration of $MgSO_4$ to 0.09 *M*. Phosvitin was precipitated as a sticky mass which settled at the bottom of the beaker when kept at room temperature for few hours. After siphoning off the supernatant, the precipitate was washed 2–3 times with water, suspended in water and dissolved by the addition of 2 g ethylenediaminetetraacetate (disodium salt). The protein solution was dialysed in the cold against several changes of distilled water. Phosvitin was precipitated as before by acidification to pH 1.8. The protein was centrifuged off, washed twice with 50 % acetone and then with pure acetone. After a final washing with ether, the product was air-dried. It was obtained as a light white powder and the yield was the same as that obtained by the procedure of MECHAM AND OLCOTT[2].

Phosphorus and nitrogen analyses of the proteins were carried out by the method of FISKE AND SUBBAROW[7] and KOCH AND MCMEEKIN[8] respectively. Vitellin had a P content of 0.66 % and contained less than 0.05 % of hot alcohol–ether extractable solids. The value for phosphorus is lower than those reported by the earlier workers whose preparations had obviously been contaminated with varying amounts of phosvitin[1,4].

Phosvitin had a N/P molar ratio of 2.65 in close agreement with that obtained for the preparation of MECHAM AND OLCOTT[2]. The present preparation and that of MECHAM AND OLCOTT were examined by paper electrophoresis in buffers of different pH. Both the samples gave a major ninhydrin-positive band, when sprayed with the acidic ninhydrin reagent of LEVY AND CHUNG[9], together with two faint slower-moving components. The most rapidly moving component reacted for phosphate when tested by the method of HANES AND ISHERWOOD[10].

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Differences between oxosteroids in their behaviour towards the Girard reagent

The formation of water-soluble derivatives of oxosteroids by treatment with Girard's reagent T is frequently utilized to effect the separation of these steroids from non-ketonic material in lipid extracts of urine[1,2] and tissues[3]. The free oxosteroids can be regenerated from such derivatives by acid hydrolysis. Recently, this procedure has been adapted to the purification of certain highly labile carbonyl compounds by the introduction of two modifications[4]: the use of a carboxylic resin instead of glacial acetic acid as catalyst for the condensation reaction, and treatment with formaldehyde in neutral solution, replacing acid hydrolysis, as a means of splitting the Girard complexes. It has been suggested[5] that the modified procedure may be applied with advantage to oxosteroids, since it eliminates the risks of causing acetylation of hydroxysteroids and partial destruction of labile compounds.

A number of steroids were subjected to the new method of separation in this laboratory. Crystalline steroids (20–200 μg) were dissolved in 1 ml ethanol containing 20 mg Girard reagent T and 5 mg of the resin Amberlite IRC-50 [H] (Rohm & Haas), and either heated to boiling under reflux for 1 h (Series I), or allowed to stand at room temperature for 12 h (Series II). After filtration and dilution with 10 ml water, the "non-ketonic fraction" was separated by extracting 3 times with an equal volume of ethyl acetate. 2 ml 36 % (w/v) formaldehyde solution were then added to the aqueous phase, which was allowed to stand for 7 h at room temperature before re-extracting with ethyl acetate to obtain the "ketonic fraction A". The aqueous residue was then acidified by the addition of 0.4 ml 10 *N* HCl or of 1.5 ml pyruvic acid, and 5–7 h later extracted with ethyl acetate to yield the "ketonic fraction B". The fractions thus obtained were washed to remove residual acid and formaldehyde and taken to dryness. The steroids were then separated by paper chromatography[6] and determined individually.

The experiments, results of which are summarized in Tables I and II, showed that the steroids examined differed markedly both in the rate of formation of a Girard derivative and in the ease with which these complexes could be split. The 3-oxo group of α,β-unsaturated 3-oxosteroids combined readily with the Girard reagent

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Abbreviations: Girard's reagent T, trimethylaminoacetohydrazide hydrochloride; trivial names of steroids, as defined in Tables I and II.

TABLE I

BEHAVIOUR OF STEROIDS AFTER TREATMENT WITH GIRARD'S REAGENT AND RESIN IRC-50[H] IN BOILING ETHANOL (SERIES I)

Data give the range of values obtained for individual compounds in each group.

Compound	Recovery (%)		
	Non-ketonic fraction	Ketonic fraction A	Ketonic fraction B
Progesterone (pregn-4-ene-3,20-dione)	< 1	88–94	0–5
Testosterone (17β-hydroxy-androst-4-ene-3-one)			
Oestrone(3-hydroxyoestra-1,3,5(10)-trien-17-one)	< 2	8–13	79–86
Androsterone (3α-hydroxy-5α-androstan-17-one)			
Androst-4-ene-3,17-dione	< 1		
11β-Hydroxyandrost-4-ene-3,17-dione			

TABLE II

BEHAVIOUR OF STEROIDS AFTER TREATMENT WITH GIRARD'S REAGENT AND RESIN IRC-50(H) IN ETHANOL AT ROOM TEMPERATURE (SERIES II)

Compound	Recovery (%)		
	Non-ketonic fraction	Ketonic fraction A	Ketonic fraction B
Progesterone	< 1	86–95	0–7
Testosterone			
Corticosterone (11β,21-dihydroxypregn-4-ene-3,20-dione			
Cortisone (17α-21-dihydroxypregn-4-ene-3,11,20-trione)			
Cortisol (11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione)			
Oestrone	17–25	6–12	61–70
Androsterone			
Androst-4-ene-3,17-dione	< 1	21–28	67–74
11β-Hydroxyandrost-4-ene-3,17-dione			
17α-Hydroxypregn-4-ene-3,20-dione	< 1	60–68	28–38
Tetrahydrocortisol (3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one)	90–95	None detected	0–2

at room temperature, and quantitative recovery of free progresterone and testosterone was obtained by treatment with excess of formaldehyde, in accordance with TAYLOR's observations[5]. On the other hand, in the case of 17-oxosteroids the recoveries were small: After treatment with Girard's reagent at high temperature, amounts of oestrone, androsterone, androst-4-ene-3,17-dione and 11β-hydroxyandrost-4-ene-3,17-dione released from the Girard complexes by formalin did not exceed 13 %; the remainder could only be recovered by acid hydrolysis. A larger proportion (21–28 %) of the Girard derivatives of the 3,17-diones was amenable to decomposition by formalin treatment if the condensation had been carried out at room temperature; this increase in the formalin-labile fraction probably reflects incomplete coupling of the 17-oxo group of these steroids with Girard's reagent under the milder conditions, as suggested by the presence of 17–25 % of androsterone and oestrone in the "non-ketonic fraction" after similar treatment.

The percentage of cortisone, cortisol or tetrahydrocortisol recovered after treatment with the Girard reagent at high temperature was small; of the total amount recovered, the larger portion was released only by acid hydrolysis. In contrast, the Girard complexes of cortisone or cortisol prepared by the milder procedure (Series II) were readily split by formalin. The difference must be due to inability of the dihydroxyacetone side-chain to combine with Girard's reagent at room temperature, since the bulk of tetrahydrocortisol was recovered from the "non-ketonic fraction" under these conditions. At the same time, the difference observed between the rates of hydrolysis of the Girard derivatives of progesterone and of 17α-hydroxypregn-4-ene-3,20-dione on addition of formalin (Table II) suggests that 21-deoxysteroids, unlike the preceding group, combine at room temperature with Girard's reagent at C-20, and that the complex so formed is more resistant to hydrolysis if a hydroxyl group is present in position 17.

It was noticed that the treatment of corticosterone, cortisol, and 11β-hydroxyandrostenedione with aq. formaldehyde gives rise to small amounts (2 %) of artefacts, appearing on Bush-type paper chromatograms[6] as u.v.-absorbing components more polar than the corresponding parent compounds. The derivatives resemble the respective parent compounds as regards the presence of an α-ketolic side-chain or of a 17-oxo function. The 11β-hydroxy group appears to be implicated in the production of these artefacts, since closely related compounds lacking such a group, *e.g.* cortisone and androstenedione, fail to produce them.

Early work[1,3] has demonstrated that the Girard complexes of αβ-unsaturated 3-oxosteroids are more resistant to acid hydrolysis than those formed by the saturated 3-oxo-5α steroids. The present study indicates that further differences exist between oxosteroids in their reactions with the Girard reagent, depending on the location of carbonyl groups and the presence of substituents in neighbouring positions; these differences largely determine which procedure is best suited for the purification of any particular class of steroids by means of this agent. While it can be confirmed that a number of oxosteroids, such as progesterone and testosterone, can be conveniently separated from non-ketonic material by use of the mild and selective method adopted by TAYLOR[5], our experiments show that it is not applicable to oxosteroids in general; in particular, it cannot be used for the purification of urinary 17-oxosteroids.

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Malate synthetase in germinating seeds

The enzymic condensation of glyoxalate with acetylCoA to yield malate (malate synthetase reaction) has been demonstrated in acetate-grown *Escherichia coli*[1] and *Pseudomonas fluorescens*[2]. Studies involving the incorporation of [^{14}C]acetate into malate have implicated this reaction in the process of conversion of fat to carbohydrate in germinating high-fat seeds[3, 4]. It is the purpose of this note to demonstrate conclusively the malate synthetase reaction in peanut extracts and further to support its participation in the process of fat conversion by a distribution study in various germinated seeds. Data will also be presented on the substrate specificity of the enzyme.

When an extract of the cotyledons of peanuts germinated for 5 days was incubated with glyoxalate and an acetylCoA-generating system (acetyl phosphate, CoA and transactylase) there was a disappearance of glyoxalate and acetyl phosphate with concomitant accumulation of malate. As shown in Table I, omission of either the enzymic or substrate components results in the absence of the reaction. The requirement for glutathione is solely for the generation of acetylCoA since subsequent experiments with acetylCoA (Fig. 1) showed no such requirement. Malate was further indentified as the product of the reaction by chromatography in butanol saturated with HCOOH.

TABLE I

MALATE SYNTHETASE OF PEANUT EXTRACT

In Expt. 1, the complete system contained 6.5 μmoles glyoxalate, 7.5 μmoles acetyl phosphate, 150 μmoles Tris buffer, pH 7.5, 50 μmoles KCl, 0.08 μmole CoA, 5 μmoles $MgCl_2$, 2.5 μmoles glutathione, 5–10 units transacetylase and 0.06 ml peanut supernatant (5-day-germinated peanuts blended for 1 min with 1.5 vol. of 0.1 *M* Tris, pH 8.0, filtered through cheese-cloth and centrifuged for 15 min at 12,000 × *g*), in a final vol. of 1.2 ml. Two sets of vessels were incubated for 20 min at 30°. One set was then deproteinized with trichloroacetic acid and glyoxalate was determined as the 2,4-dinitrophenylhydrazone[6]. The second set was deproteinized by heating for 2 min at 100° and malate was determined with malic dehydrogenase. The method involves addition of an aliquot containing less than 0.15 μmole malate to 2.0 ml glycine–semicarbazide (0.2 *N* glycine containing 10 g semicarbazide·HCl/100 ml adjusted to pH 10.4 with NaOH), and 0.5 μmole DPN in a vol. of 3.0 ml. The absorbance at 340 mμ is noted and 0.03 ml of an $(NH_4)_2SO_4$ suspension of malic dehydrogenase (Worthington Biochemical) is added. The change in absorbance at 340 mμ is again noted after 6 h. Under these conditions the increase in absorbance is proportional to malate up to 0.15 μmole with an absorbance change of 0.128 for 0.1 μmole. With these concentrations there is no further change in absorbance at 340 mμ after 6 h. Expt. 2 was similar to Expt. 1 except for 9.6 μmoles glyoxalate, 5.0 μmoles acetyl phosphate and 150 μmoles of Tris buffer, pH 8.0, and 0.05 ml of peanut supernatant. Acetyl phosphate was determined by the hydroxamate reaction[7]. The results are corrected for non-enzymic glyoxalate disappearance and acetyl phosphate hydrolysis by acetyl phosphatase in the peanut extract.

	Expt. 1		*Expt. 2*
Conditions	*Glyoxalate disappeared (μmoles)*	*Malate formed (μmoles)*	*Acetyl phosphate disappeared (μmoles)*
Complete system	1.8	2.2	1.6
No peanut enzyme	0.0	0.0	0.0
No transacetylase	0.1	0.1	0.0
No CoA	0.2	0.1	0.1
No $MgCl_2$	—	—	1.4
No glutathione	0.2	—	0.2
No glyoxalate	—	0.2	0.0
No acetyl phosphate	0.1	0.1	—

Abbreviations: CoA, coenzyme A; Tris, tris(hydroxymethyl)aminomethane.

Examination of extracts of the cotyledons of germinated castor beans and soybeans showed that only 10 to 20% of the activity could be extracted into the supernatant by blending in either phosphate or Tris buffer. Accordingly, a distribution study was performed with whole homogenates of various tissues. The results (Table II), showing that the enzyme is present in large amounts only in high fat seeds, lend support for the function of malate synthetase in the utilization of fat. The enzyme is

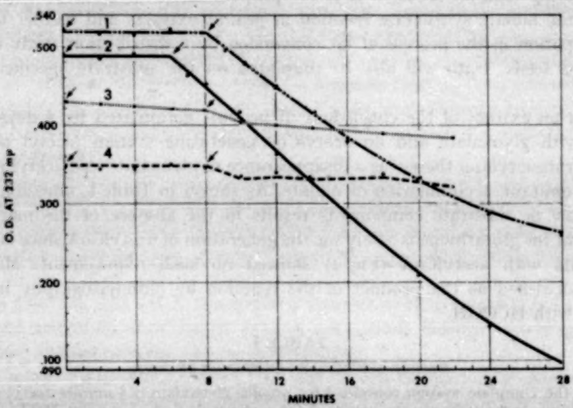

Fig. 1. All experimental cuvettes contained 100 μmoles Tris buffer, pH 8.0, 5 μmoles $MgCl_2$, acyl thiolester and water to 1.0 ml. Cuvettes 1 and 2 contained, respectively, 0.1 and 0.2 μmole acetylCoA. Cuvettes 3 and 4 contained, respectively, 0.1 μmole acetyl glutathione and acetyl pantetheine. The blank cuvette contained sufficient acetyl pantetheine to bring the experimental cuvette to the desired absorbance. At the first arrow in cuvettes, 1, 3, and 4, 0.02 ml of 6-day-peanut supernatant (diluted 10-fold with water) was added and at the second arrow 0.01 ml 0.12 *M* potassium glyoxalate was added. In cuvette 2, the order of addition was reversed. All values are corrected for absorbance due to the component added. All acyl thiolesters were prepared by the method of SIMON AND SHEMIN[8].

TABLE II

DISTRIBUTION OF MALATE SYNTHETASE

The assay system was that described in Table I, Expt. 1. Under these conditions glyoxalate disappearance is proportional to enzyme concentration up to 1.6 μmoles removed. The chicken-liver and pig-heart preparations were 10% extracts of acetone powders in 0.1 *M* Tris, pH 8.0. All other preparations were homogenates in 1.5 vol. 0.1 *M* Tris, pH 8.0, filtered through cheese-cloth. The seeds were germinated for 5 days on wet towels at appropriate temperatures. The sunflower roots were obtained from a 5-day seedling and the leaves from a young field plant. Barley embryos were tested after overnight standing on moist filter paper. At least 3 points within the range of linearity were obtained for each sample.

Material	*μmoles/ml/h*	*Material*	*μmoles/ml/h*
Sunflower seed	156	Barley seed	0
Cotton seed	144	Barley embryo	0
Peanut seed	91	Kidney bean seed	0
Castor bean seed	84	Sunflower root	1.2
Soybean seed	48	Peanut leaves	0
Pea seed	14.1	Chicken-liver extract	0
Corn seed	6.0	Pig-heart extract	0

apparently restricted to the cotyledon as several analyses of leaf and root homogenates showed no activity. In experiments not shown here, it was found that the enzyme is present in extracts of acetone powders of eithers peanut or castor-bean cotyledons.

In order to test the specificity of the malate synthetase reaction, various compounds were compared with acetyl phosphate in the assay system of Table II. With acetyl pantetheine, acetyl glutathione and fluoroacetyl phosphate[5], 0.1, 0.4, and 0.1 μmoles glyoxalate disappeared. The data with fluoroacetyl phosphate and acetyl pantetheine are within experimental error while the apparent activity with acetyl glutathione may have been due to liberation of free thiol by a thiolesterase in the extract, with subsequent non-enzymic condensation with glyoxalate. To test this point, the reaction was followed by disappearance of thiol ester at 232 mμ. As shown in Fig. 1 neither acetyl pantetheine nor acetyl glutathione are reactive in the malate synthetase reaction and the extract does contain acetyl glutathione thiolesterase. These experiments further establish that acetylCoA is the reactive component and that both glyoxalate and enzyme are required for the reaction. In similar spectrophotometric experiments it was found that both propionylCoA and butyrylCoA are not active, indicating complete specificity of the reaction for acetylCoA.

Two types of experiments were performed to test the reversibility of the reaction (a) incubation of malate, CoA, and enzyme with hydroxylamine and analysis for acethydroxamic acid (b) incubation of malate, CoA and enzyme with a system of removal of acetylCoA (transacetylase and arsenate) and analysis for glyoxalate. The negative results obtained in both experiments indicate that the equilibrium is far in the direction of malate and that detection of reversibility will require an extremely sensitive assay.

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A method for plotting ultracentrifuge diagrams for polydisperse systems

The results of ultracentrifugal analyses, as generally recorded photographically, show a plot of the rate of change of refractive index (dn/dx) *versus* the distance from the center of rotation (x). To show changes occurring with time, several pictures are necessary. We therefore decided to examine the alternate possibility of using a plot of dn/dx *versus* time at one value of x since the results of one ultracentrifugal analysis may thus be presented in one picture. This is of some advantage where, by the usual method, a number of diagrams from each analysis would be necessary to show both fast and slow peaks and several rows of diagrams would be needed for comparison of different samples. Results obtained by the usual method may be confusing since it is sometimes difficult to intercompare different runs, especially where the time intervals are not uniform.

A swing-out mask with a 0.03-inch vertical slit was installed close to the focal plane of a Spinco Model E analytical ultracentrifuge. The photographic plate was moved 0.05 inch every 2 min by a short-shift assembly. Two identical 12-mm 4° ultracentrifuge cells were used, one containing the solution to be analyzed, the other the solvent to provide a reference base line. Small differences between cells may be compensated by alternating the two solutions between the cells in consecutive runs.

Less exposure was needed to record the position of the base line than the experimental pattern. In these experiments the amount of light passed by the base-line cell was reduced by adding a small amount of the dye cloth red B to increase the extinction to about 0.25 at 580 mμ (1-cm light path), The Eastman Kodak Metallographic plates used were developed for 5 min in DK-19 developer. All runs were made at 56,100 rev./min and at 20° with a type D analytical rotor.

The first exposure was made, without the mask in place, as soon as the rotor had reached the desire speed. The mask was then moved into place and a second exposure made without moving the photographic plate. In this manner the position of the slit in relation to the meniscus and the reference hole was accurately located. The plate was then moved about 4 cm and exposures at 2-min intervals were begun.

The results of an ultracentrifuge run in which rat serum diluted 1:3 with buffer and dialyzed against buffer (pH 7.5, I 0.2) containing both phosphate and NaCl[1] were used are shown in Fig. 1A as a series of photographs* made at the indicated intervals. The results obtained on a similar preparation with the method described here are shown in Fig. 1B. In this experiment the level in the cell corresponding to the center of the slit was 0.535 cm from the meniscus.

The sedimentation coefficient of a given peak is inversely proportional to the time required for it to reach the slit level from the meniscus. The difficulty is in determining the true zero or starting time at which a given peak left the meniscus. In one series of experiments the centrifugation during acceleration was found by determining the area under a centrifugal force *versus* time curve for this period and calculating from

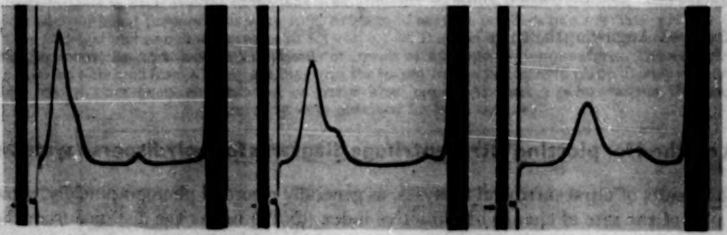

Fig. 1. A. Rat serum albumin diluted 1:3 with buffer (pH 7.5, I 0.2), photographed 20, 44, and 92 min after reaching 56,100 rev./min. Bar angle 55°, 20°C.

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* Unfortunately the short shift assembly as supplied by the manufacturer moves the film the wrong direction. The photographic print must therefore be cut up into strips and reassembled.

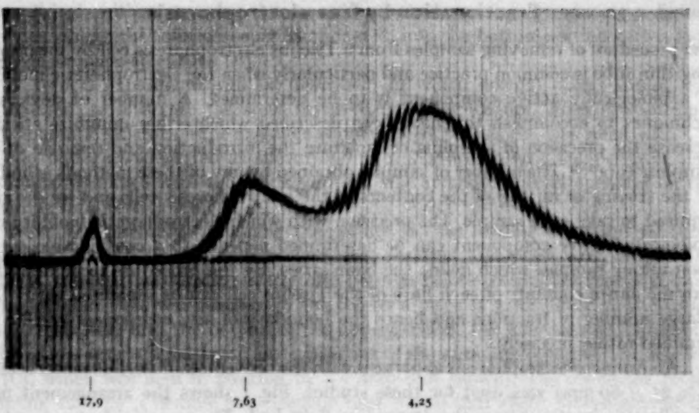

Fig. 1. B. Similar preparation photographed by method described in text. Bar angle 50°, 56,100 rev./min. Photographs taken at 2-min intervals.

this the time at operating speed producing an effect equal to that incurred during acceleration. Subtracting this time from the time at which the first picture was taken gave a value for zero time. When the distance from the meniscus to the peaks (measured on conventional photographs of the Schlieren diagram) was plotted against time, the lines obtained did not converge exactly at the meniscus level at zero time for the evident reason that a period of time is required to form the boundary at the meniscus. The method described[1] is therefore subject to this error. Considerable improvement in accuracy could be obtained by using a synthetic boundary cell to form the boundaries and a continuous film drive combined with a very narrow slit to obtain a smoother curve.

In Fig. 1B the photographs taken through the slit are spaced evenly along a time scale. Since the less rapidly sedimenting proteins will pass the slit more slowly, their peaks will occupy a larger area than more rapidly sedimenting peaks produced by the same concentration of protein. The area under each peak will therefore not be proportional to the concentration of the constituents unless the area is divided by the elapsed time.

Since the chief use of this method will probably be illustrative, the sedimentation coefficients may be determined in separate experiments and used to calculate the values for the abscissa as has been done in Fig. 1B.

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Fractionation by free electrophoresis

The procedure of removing samples from a Tiselius electrophoresis cell by means of a long thin tube is common practice and particularly when the electrophoretic mobility of a biologically active component is to be determined. A number of devices or techniques to accomplish this have been described which either minimize stirring, increase the precision of sampling or facilitate the introduction and removal of the sampling tube[1–3]. The number of samples obtained by any of these methods is limited by the stirring or mixing of the contents of the electrophoresis cells and by the time required to take each sample. The precision with which electrophoretic mobility of a biologically active component can be determined depends to a considerable extent upon taking samples which define the boundaries. By the procedure to be described here the entire contents of each limb of the Tiselius cell can be separated easily into a large number of fractions and hence the boundary of any component can be determined rather precisely.

An Aminco electrophoresis apparatus with the conventional Tiselius center section (3 × 25 × 80 mm) was used for these studies. Fig. 1 shows the arrangement used for collecting fractions from the descending (left) limb. The essential features are the 18-gauge stainless-steel tube (A) extending into the center section and a method of forcing the solution in the descending column up the steel sampling tube. This was

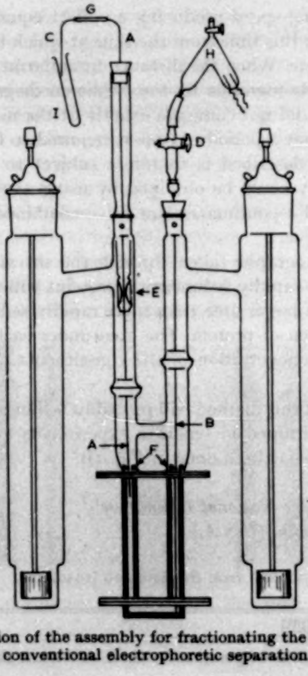

Fig. 1. A diagrammatic section of the assembly for fractionating the descending (left) limb after a conventional electrophoretic separation.

accomplished, without moving the ascending boundaries, by introducing a liquid, more dense than the solution being fractionated, into the bottom section with a long 22-gauge stainless-steel tube (B).

A typical experiment will be described, in which normal rabbit serum was fractionated, to illustrate the steps involved in the procedure. After conventional electrophoretic separation the 22-gauge stainless-steel tubing (B) was filled with 5% NaCl in buffer and inserted as shown in Fig. 1. The electrolytic compensator (H), which also was filled with the saline solution, was attached and all openings on the ascending side were closed.

Next, the assembly containing the springs (E) for holding the sampling tube, which has been described by HECKLY[4], was inserted and filled with buffer. The sampling tube (A) was pushed nearly into position before seating the rubber stopper at the top. The tube was centered in the cell by a piece of 7 mm thick plastic which was fitted loosely into the bottom of the upper section. The hole through the center of the plastic was tapered to facilitate insertion of the sampling tube (A). The delivery tip (C), which was held in position by a clamp (not shown), was connected to the sampling tube by a flexible polyethylene tube (G) so that slight movements of the delivery tip would not be transmitted to the sampling tube which would mix the cell contents.

The current through the electrolytic compensator was set at 10 mA, the collecting tube was changed at 1-min intervals, and photographs were taken at 5-min intervals. The photographic record was used to determine the position of the sampling tube, the time of entry of each component into the tube, and to relate the fractions to cell position. The rate of movement of the albumin peak, determined from the photographic record, indicated that the volume delivered to each collecting tube was approximately 0.104 ml. Obviously, lower feed rates and a larger number of fractions can be taken to improve the resolution. It is important, particularly with smaller fractions, that the delivery tip be made to touch the wall of the collecting tube at all times to minimize variations in flow rate due to pressure differences produced by surface tension. The maximum allowable delivery rate was not determined, but the 18-gauge tubing did not produce excessive back pressure at a flow rate of 0.3 ml/min. If the pressure becomes appreciable, there is a danger of unseating a joint or a part of the Tiselius cell. It was found that the hold up in the sampling-tube assembly was approximately 0.4 ml, and, therefore, the contents of collecting tube No. 5 correspond to the material which was at the lower end of the sampling tube when fractionation was started.

The procedure for fractionating the ascending limb was comparable to that described above, except that buffer was introduced in the upper section of the descending limb. The 5% NaCl previously introduced into the bottom section was adequate to stabilize the cell contents.

Fig. 2 shows the results of an experiment in which the components of normal rabbit serum in 0.1 *M* veronal buffer, pH 8.5, were separated electrophoretically. The typical schlieren pattern, which is a measure of the refractive-index gradients in the column, is shown below the curve indicating the protein concentration of the fractions. The two are presented on a common scale to facilitate direct comparisons, each fraction representing 1.4 mm of cell height.

The protein content was determined by the method of LOWRY *et al.*[5], using bovine serum albumin as the standard. A comparison of the two curves shows that each step in the protein curve corresponds to one of the protein peaks of the schlieren pattern. It is also apparent that the concentration of each component can be estimated from the protein curve. For example, the protein concentration of fraction No. 42 was 5 mg/ml, which represents the globulins, and the protein concentration increased from 5 to 13.5 mg/ml between fractions No. 43 and 53 (the albumin component); hence, the preparation contained approximately 5 mg/ml globulin and 8.5 mg/ml albumin.

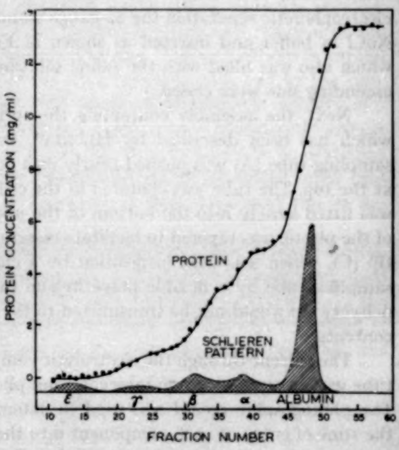

Fig. 2. Electrophoretic patterns of a normal rabbit serum in 0.1 *M* veronal buffer, pH 8.6 after 140 min at 7.4 V/cm. The upper curve represents the protein concentration of each fraction obtained from the descending limb, and the lower is a tracing of the usual schlieren pattern as it appeared before fractionation. The flow rate was 0.1 ml/min, and each fraction represented about 1.4 mm of cell height.

The described method makes practical the measurement of the electrophoretic mobilities and concentrations of biologically active components independent of the schlieren pattern and is of great value when working with highly biologically active materials whose concentration is too low to be detected by the optical methods. A significant advantage of free boundary electrophoresis over paper electrophoresis is that one need not be concerned with adsorption and the mobilities can be measured directly without comparison with a substance of known mobility. Thus, fractionation of the entire contents of both limbs facilitates electrophoretic studies of active materials and increases the precision with which the mobility can be determined.

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Avoidance of alkaline effects in cell disruption by glass beads

Small glass beads (ballotini), in conjunction with mechanical shaking, sonic oscillation and grinding, have been found a convenient means for the disruption of cells preparatory to the study of the soluble constituents[1]. In our own experience with a low-temperature, high-speed shaking procedure for the disruption of bacteria[2] we have noted many cases of alkaline reaction, approaching pH 10, in the resulting suspensions. Because of the alkali sensitivity of many biochemical substances (phosphoric acid esters, ribonucleic acid, etc.) we have studied means of overcoming the alkaline effect which is obviously attributable to glass solubilization.

Repeated shaking of 25 g of 0.2-mm beads with an equal weight of distilled water for 25 min in chrome-plated, stainless-steel capsules at 1,800 rev/min with the centrifuge compressor maintaining —5° (so that the temperature within the capsule was between 0 and 5°) showed in consecutive supernatants mean pH values of 10.2, 10.0, 9.9, and 9.8. Therefore, depletion of the surface alkalinity of the glass in this way did not seem attractive. Extended heating with strong HCl or HNO_3 was equally ineffective.

We have examined 11 varieties of beads of different manufacturers and among these we have found only one kind which does not impart significant alkalinity to water. We, therefore, recommend these or equivalent beads for cell disruption where alkali sensitivity may be a factor. They are beads of refractive index 1.68, made by Prismo Safety Corporation, Huntingdon, Pa. These beads can be cleaned by shaking with water (or detergent if necessary) followed by several washings with 95 % ethanol and drying at 120°. Direct drying by heat of water-washed beads seems to damage the surface, as indicated by a tendency toward alkaline pH when used. These beads partially dissolved in strong acids and therefore cannot be washed in this manner. The "Prismo" beads, when shaken with distilled water of pH 6.0 and the type of bacterial preparations previously described by us[3], typically produced suspensions and supernatants of pH 6.6 to 6.8. We have also tested these beads by u.v. measurements for their propensity to yield soluble impurities. After shaking equal weights of water and beads for 20 min, the absorbance of the filtrates was 0.14, 0.04, and 0.04 at 215, 230 and 260 mμ respectively; corresponding to less than 0.1 % of the extinction values of typical bacterial extracts, which are read at dilutions of about 1:200.

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Many thanks to Mr. J. E. Barefoot of Jefferson Medical College for suggesting the Prismo Safety Corporation as the source of the glass beads now used in our work.

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Preliminary Notes

Inhibition of 3α-hydroxysteroid-mediated transhydrogenase of rat-liver homogenate by Δ^4-3-oxosteroids

The 3α-hydroxysteroids with saturated ring A, *e.g.*, androsterone, and 17β-hydroxysteroids, *e.g.*, estradiol, have been shown to function *in vitro* as co-enzymes for hydrogen transfer in enzyme systems[1-3]. We have found an apparent inhibition of 3α-hydroxysteroid-mediated transhydrogenase from rat-liver homogenate by Δ^4-3-oxosteroids, *e.g.*, testosterone. The present report deals with the structural specificity of the inhibitory steroids.

A 3α-hydroxysteroid dehydrogenase was prepared as a 50–70 % ammonium sulfate fraction of adult-male-rat liver by the method of HURLOCK AND TALALAY[2]. Preparations obtained from adult-female-rat livers were also active. 3α-Hydroxysteroids and 3-oxosteroids with a saturated ring A mediated hydrogen transfer at rates comparable to those previously reported[2]. In this study androsterone ($4 \cdot 10^{-6}\,M$) was the principal steroid used to mediate transhydrogenation. Testosterone was purified extensively by chromatography, recrystallization, and sublimation. The purified testosterone dissolved in dioxane was added to the reaction vessel either at the onset or 10 min after initiation of hydrogen transfer with androsterone (Fig. 1). Inhibition of transhydrogenation occurred in both instances. Testosterone also inhibited hydrogen transfer mediated by androstane-3,17-dione, 5β-androstane-17β-ol-3-one, 5β-androstane-3,17-dione, and 5α-pregnane-3α,20α-diol.

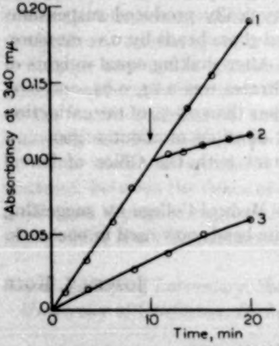

Fig. 1. Testosterone inhibition of androsterone-mediated transhydrogenase. The complete system contained in a volume of 3 ml: 200 μmoles tris(hydroxymethyl)aminomethane buffer, pH 7.5, 1 μmole DPN, 10 μmoles glucose 6-phosphate, 1 μmole $MgCl_2$, excess of glucose 6-phosphate dehydrogenase, 0.03 μmole TPN, 3 μmoles ethylenediaminetetraacetic acid and 0.1 ml (6.3 mg protein) of 50–70 % ammonium sulfate fraction of rat-liver homogenate. 3.5 μg androsterone in 0.01 ml dioxane were added to initiate the reaction. 3.3 μg testosterone in 0.01 ml dioxane were added at times 0 and 10 min to vessels 3 and 2, resp. Measurements were made of absorbancy at 340 mμ against a control cuvette containing all ingredients except steroid. Temp. 25°.

All steroids tested that had a 3-oxo group and conjugated double bond were effective inhibitors of the androsterone-mediated transhydrogenase. Greatest inhibition was obtained with $\Delta^{1,4}$-3-oxosteroids (1-dehydrotestosterone and 1-dehydrotestololactone), complete inhibition occurring at a concentration of $1 \cdot 10^{-6}\,M$. The

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Abbreviations: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide.

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inhibitory concentration was less than that of androsterone ($4 \cdot 10^{-6} M$) used to mediate hydrogen transfer. An inhibitory influence was noted at a concentration as low as $3 \cdot 10^{-7} M$. In diminishing order the inhibitory potency of the Δ^4-steroids was: 3-oxo > 3α-hydroxy > 3β-hydroxy. Estradiol, 17α-ethyl-19-nortestosterone, hydrocortisone, and 11-dehydrocorticosterone were less effective inhibitors than 4-androstene-3,17-dione. The inhibitory influence produced by progesterone and 11-deoxycorticosterone was transitory with recurrence of hydrogen transfer after 10 min. The recurrence was probably due to saturation of Δ^4-bond by the Δ^4-3-ketosteroid hydrogenases in the system[4–7]. Tomkins[8] reported that α,β-unsaturated steroids inhibited 3α-hydroxysteroid dehydrogenase of rat-liver homogenate.

The degree of inhibition of 3α-hydroxysteroid-mediated transhydrogenation correlated with the quantity of testosterone added. The extent of inhibition was greater when testosterone was added 10 min after mediation of the reaction than at the start. Testosterone inhibition of androsterone-mediated transhydrogenase depends on the relative concentration of DPN and TPN. Preliminary study indicates that a testosterone-dependent inhibitory substance may be present in the rat-liver homogenate.

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Cellulose polysulfatase, an enzyme attacking cellulose polysulfate and charonin sulfate

An enzyme hydrolysing sulfuric ester bonds in cellulose polysulfate and charonin sulfate has been found in the liver extract of a marine gastropod, *Charonia lampas* (*Tritonalia sauliae*). Since chondroitin sulfate and amylose polysulfate are scarcely hydrolysed by the enzyme preparation, we propose to name the new sulfatase "cellulose polysulfatase".

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The liver extract contains, besides cellulose polysulfatase, polysaccharases including cellulase and glucosulfatase, a sulfatase hydrolysing glucose sulfate and other sulfate esters of carbohydrates of low molecular weight[1]. The degradation of cellulose polysulfate by the enzyme preparation proceeds as follows:

$$\underset{\text{(S-rich)}}{\text{Cellulose polysulfate}} \xrightarrow[\text{Cellulose polysulfatase}]{} \underset{\text{(S-poor)}}{\text{Cellulose sulfate}}$$

$$\xrightarrow[\text{Polysaccharases}]{} \text{Glucose sulfates and glucose} \xrightarrow[\text{Glucosulfatase}]{} \text{Glucose}$$

Potassium cellulose polysulfate and potassium amylose polysulfate were prepared as usual by sulfation of cellulose and amylose respectively with chlorosulfonic acid in pyridine. (S contents: cellulose polysulfate, 17.5 %; amylose polysulfate, 15.75 %). Potassium charonin sulfate was prepared from the mucous gland of *Charonia lampas* according to EGAMI *et al.*[2] (S content, 16.2 %). It consists mainly of cellulose polysulfate. Sodium dextran polysulfate prepared as an artificial anticoagulant was kindly supplied by Dr. S. SASAKI[3] (S content, 17.7 %). Commercial sodium chondroitin sulfate (K. K. Seikagaku Kenkyusho) from shark cartilage was used (S content, 6.03 %).

The enzyme solution was prepared by homogenizing fresh liver (12 g) of *Charonia lampas* in 48 ml 0.1 *M* acetate buffer, pH 5.2, and centrifuging. The 100,000 × *g* supernatant, after dialysis, was used.

Unless otherwise mentioned, the enzyme activities were determined with a reaction mixture containing 2.5 ml enzyme solution, 15 mg polysaccharide sulfate (25 mg in the experiment of Table I), adjusted to 5 ml with 0.1 *M* acetate buffer, pH 5.2. A few drops of chloroform were added and the reaction mixture was incubated at 37°. Sulfatase activity was determined by estimating liberated inorganic sulfate by the method of DODGSON AND SPENCER[4] with the following pretreatment. To 1 ml of the reaction mixture, 2 ml of 1 % trypaflavin solution was added to precipitate polysaccharide sulfates in the reaction mixture. To 2 ml of the supernatant, ethanol, trichloroacetic acid and benzidine solution were added as described by DODGSON AND SPENCER. Polysaccharase activity was determined by estimating reducing power by the method of FOLIN AND MALMROS[5] after deproteinization with cadmium hydroxide gel.

As shown in Table I, charonin sulfate and cellulose polysulfate were rapidly desulfated, and dextran polysulfate only slowly. Amylose polysulfate and chondroitin sulfate were scarcely attacked.

TABLE I

SPECIFICITY OF CELLULOSE POLYSULFATASE

Substrates	*Liberated sulfate (%) after*		
	24 h	*72 h*	*120 h*
Charonin sulfate	38.1	74.6	92.9
Cellulose polysulfate	25.7	41.6	65.4
Dextran polysulfate	2.7	8.2	13.0
Amylose polysulfate	0	0	0.5
Chondroitin sulfate	0	0	0

As shown in Table II, 0.02 *M* sodium fluoride completely inhibited polysaccharase, but only slightly cellulose polysulfatase.

Charonin sulfate and cellulose polysulfate were digested for 3 days under the usual experimental conditions. 72.6 % of the sulfate and 57.2 % of the reducing power were liberated in the experiment with charonin sulfate, and 64.2 % and 29.1 %, respectively, in the case of cellulose polysulfate. For the identification of the reaction products, both reaction mixtures (each 4 ml) were boiled and centrifuged. To the supernatants, 400 mg of norit pretreated with *N* HCl were added, stirred and centrifuged. The sediments were washed 3 times with 2 ml water, then eluted several times with 5 % aq. pyridine[6]. The eluates were dried in the cold and subjected to paper chromatography with glacial acetic acid–*n*-butanol–water (15 : 50 : 35) as solvent[7], developed with aniline phthalate. Only one spot, identified with that of glucose, was obtained from both reaction mixtures. Neither disaccharides nor glucose sulfates were found to be present. On the other hand, in the presence of fluoride, S-poor cellulose sulfate was obtained. Cellulose polysulfate was incubated with the enzyme solution for 3 days as described above but with 0.02 *M* sodium fluoride. No increase of reducing power was detected during the incubation. After deproteinization, the reaction mixture was thoroughly dialyzed to remove inorganic sulfate, and ester sulfate and glucose in the non-dialyzable product were estimated. The ratio $\frac{\text{ester sulfate}}{\text{glucose}}$ was found to be 0.65. After further digestion of the product with fresh enzyme solution the ratio decreased to 0.5. Thus, the final product may be regarded as cellulose sulfate containing, on the average, one sulfate group for each cellobiose unit.

TABLE II

EFFECT OF FLUORIDE ON POLYSACCARASES AND CELLULOSE POLYSULFATASE

Time (h)	*Liberated sulfate (%) from cellulose polysulfate*		*Liberated reducing power (% estimated as glucose for the complete hydrolysis)*	
	No inhibitor	*+ NaF*	*No inhibitor*	*+ NaF*
24	68.5	43.4	20.1	0

The purification of cellulose polysulfatase and the separation from polysaccharases are now in progress.

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Enzymic dehydrogenation of steroid A ring

Cell-free extracts of a *Nocardia sp.** have been found capable of introducing double bonds into the 1,2- and 4,5-positions of steroids possessing either the 5β-pregnane or 5α-pregnane nucleus.

Cells of this organism were grown on the following medium: corn steep liquor, 0.6 %; $NH_4H_2PO_4$, 0.3 %; $CaCO_3$, 0.25 %; soybean oil, 0.22 %; yeast extract, 0.25 % and glucose, 1 %. After 72-h growth, the cells were harvested by centrifugation and washed with cold 0.03 *M* phosphate buffer (pH 7.0). Cell-free extracts were prepared by placing a suspension of cells in the sonic field of a Raytheon 10 kc magnetostrictive oscillator for 30 min. The cell debris was removed by centrifugation at 100,000 × *g* for 30 min; the supernatant containing about 6 mg protein/ml was used as the source of enzymes.

In the presence of a suitable electron acceptor such as phenazine methosulfate, the cell-free extracts of this organism carried out efficient conversions of pregnane-3,20-dione, 5α-pregnane-3,20-dione and progesterone to $\Delta^{1,4}$-pregnadiene-3,20-dione. In a typical experiment, 200 mg pregnane-3,20-dione in 5 ml dimethyl formamide were incubated at 25° with 150 mg phenazine methosulfate and 200 ml of enzyme solution in a total volume of 400 ml in 0.03 *M* phosphate buffer, pH 7.0. After 10-h incubation, the reaction mixture was extracted with methyl isobutyl ketone and the extract chromatographed on a cellulose-powder column (1.6·20 cm) using the hexane-propylene glycol system; 3.0-ml fractions were collected every 2.5 min. The Δ^4-3 keto-steroids were detected using the arsenomolybdate reagent of NELSON[1] with 30-min heating. Fractions 6–15 were pooled and twice rechromatographed to obtain fractions free of phenazine methosulfate. Crystallization from hexane gave about 5 mg of a substance, m.p. 122° and 129°; λ_{max} (ethanol), 241 mμ (Σ, 16,500) and an infrared spectrum identical with that of an authentic sample of progesterone. Mixed chromatography with progesterone showed a single spot. Fractions 18–25 were also pooled and crystallization from ethyl acetate-hexane afforded 90 mg of a substance m.p. 150–152°; λ_{max} (ethanol), 244 mμ (Σ, 16,500). Its infrared spectrum in Nujol exhibited bands at 5.80 μ (20 ketone), 6.04, 6.17 and 6.26 μ ($\Delta^{1,4}$, 3-ketone) identical with that of an authentic sample of pregna-1,4-diene-3,20-dione. On mixed paper chromatography, the compound had the same mobility as pregna-1,4-diene-3,20-dione.

Similar experiments were carried out with progesterone and α-pregnane-3,20-dione; in each case pregna-1,4-diene-3,20-dione was isolated and identified as described. From the evidence obtained, it appears that this organism probably contains at least 5β- and 5α-Δ^4-steroid dehydrogenases as well as a Δ^1-steroid dehydrogenase similar to the system in *Pseudonomas testosteroni* as reported by LEVY AND TALALAY[2].

A convenient and rapid method of demonstrating the enzymic activity of the Δ^1-steroid dehydrogenase is shown in Fig. 1. A reaction mixture of a Δ^4-3-oxosteroid and 2,6-dichlorophenol indophenol was incubated with the cell-free extract in a 3 ml cuvette at room temperature and the absorbancy at 600 mμ was measured with a Beckman D.U. spectrophotometer. Fig. 1 shows the rapid decrease in absorbancy by the steroid–dye mixture. Di- and triphosphopyridine nucleotides, mammalian cytochrome *c* and coenzyme Q_{10} did not act as hydrogen or electron acceptors for the Δ^1-steroid dehydrogenase.

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* This organism was isolated from a sample of corn silage by Dr. A. LASKIN of our laboratory using cholesterol as the sole source of carbon.

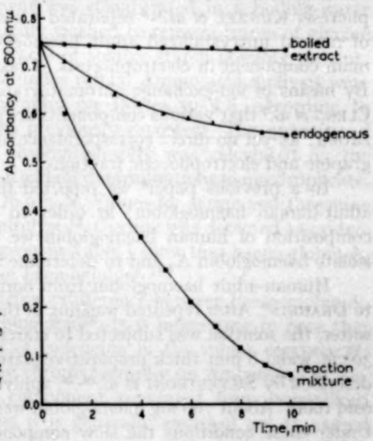

Fig. 1. The reduction of 2,6-dichlorophenol indophenol by the Δ^1-steroid dehydrogenase. The reaction mixture contained 60 μg 2,6-dichlorophenol indophenol, 100 μg progesterone and 0.2 ml enzyme in a total volume 3.0 ml in 0.03 *M* phosphate buffer, pH 7.0.

TABLE I

REACTION VELOCITIES OF Δ^1-STEROID DEHYDROGENASE WITH VARIOUS ELECTRON ACCEPTORS

The cells were broken sonically as described followed by centrifugation at 2000 × *g* for 10 min. The supernatant containing 20 mg protein/ml was the source of enzyme. The assay system consisted of 1 mg progesterone in 0.1 ml dimethyl formamide, 1 ml of enzyme solution and various concentrations of different electron acceptors in a total volume of 5.0 ml 0.03 *M* phosphate buffer, pH 7.0. After 10 min, the reaction was terminated by extraction with 1 ml methyl isobutyl ketone and chromatographed on Whatman No. 1 paper; developed in the methyl cyclohexane-carbitol system. Pregna-1,4-diene-3,20-dione was eluted with 95% ethanol and determined by absorbancy at 240 mμ. The values given here are relative maximum velocities.

Electron acceptor	*Activity* %
Phenazine methosulfate	100
2,6-dichlorophenol indophenol	50
Resazurin	40
Methylene blue	< 20
Ferricyanide	< 20

Table I shows the relative maximum velocities ($V_{\max}$) determined by the method of LINEWEAVER AND BURK[3] of a few electron acceptors with a crude enzyme preparation. Phenazine methosulfate seems to act as the best electron acceptor whereas methylene blue and ferricyanide are poor electron acceptors for the Δ^1-steroid dehydrogenase.

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Amino acid composition of human haemoglobin A_2

Haemoglobin is not a homogeneus protein[1-3]; by means of starch-block electrophoresis KUNKEL *et al.*[4,5] separated a component (Hb A_2), comprising about 2.5 % of normal uncrystallized adult haemoglobin, which moved more slowly than the main component in electrophoresis. A faster-moving component was also described. By means of ion-exchange chromatography it was shown by ALLEN *et al.*[6] and by CLEGG *et al.*[7] that various components are present in haemoglobin, even after crystallization; as yet no direct correspondence has been established between the chromatographic and electrophoretic fractions.

In a previous paper[8] we reported the amino acid composition of crystallized adult-human haemoglobin; in order to obtain a better picture of the chemical composition of human haemoglobin we have thought it of particular interest to isolate haemoglobin A_2 and to determine its amino acid content.

Human-adult haemoglobin from normal individuals was crystallized according to DRABKIN[9]. After repeated washing of the crystals in phosphate and dialysis against water, the solution was subjected to starch-block electrophoresis, using a 45 cm long, 30 cm wide, 8 mm thick preparative plate, in glycine–NaOH buffer, pH 8.8, I 0.12 as described by SILVESTRONI *et al.*[10,18], applying a potential of 300 V for 24–36 h in the cold room. About 700 mg haemoglobin were fractionated in each electrophoretic run. Under these conditions the slow component moves towards the cathode owing to the prevalence of electroosmosis, and a good separation from the main fraction is obtained. The starch zones containing haemoglobin A_2 were cut off from several such electrophoretic plates, and the pigment was eluted with water on a sintered-glass funnel. In order to obtain a better purification, the pooled A_2 fractions were lyophilized, and again submitted to electrophoresis on starch block and eluted. Haemoglobin A_2 thus obtained behaves like an electrophoretically homogeneous protein,

TABLE I

AMINO ACID COMPOSITION OF HUMAN HAEMOGLOBIN A_2

	g amino acid/100 g protein				
	I	*II*	*III*	*IV*	*average*
Aspartic acid	10.725	11.118	10.399	11.300	10.885
Threonine	4.241	4.300	4.200	4.161	4.225
Serine	5.899	6.188	6.060	5.960	6.026
Glutamic acid	7.304	7.667	7.642	7.848	7.615
Proline	4.090	3.747	4.529	3.832	4.049
Glycine	4.186	4.343	4.090	4.095	4.178
Alanine	7.504	7.783	7.343	7.709	7.584
Valine	7.449	7.354	7.544	7.149	7.374
Methionine	1.346	1.429	1.204	1.556	1.383
Isoleucine	1.019	1.145	1.095	1.199	1.114
Leucine	11.730	12.198	11.784	11.235	11.736
Tyrosine	2.744	2.969	2.656	2.904	2.818
Phenylalanine	6.574	6.417	7.016	6.379	6.596
Histidine	5.850	5.661	5.773	—	5.761
Lysine	8.650	8.470	8.667	—	8.595
Arginine	3.283	3.341	3.874	—	3.499

I, II, III and IV refer to four different analysis carried out on various amount of protein (ranging from 1.4–3 mg) from the same hydrolysate.

and migrates like the A_2 component of fresh crystalline haemoglobin analyzed on the same starch block or on paper[11].

After dialysis against water the pigment was denaturated in a boiling-water bath for 15 min; the precipitate was repeatedly washed to remove traces of glycine that could have escaped the dialysis, and traces of soluble starch possibly present; it was then hydrolized for 24 h in 6 *N* HCl under reflux. Amino acid analysis were performed in quadruplicate by chromatography on Dowex 50-X 8, according to MOORE AND STEIN[12] with the modifications previously reported[8]. The quantity of protein present in each sample used for chromatography was calculated on the basis of the N content determined by micro-Kjeldahl determinations in quadruplicate, assuming that the haemoglobin contained 16.9 % N. Values for serine and threonine were not corrected for decomposition on hydrolysis[13]. Cystine was detected as cysteic acid by paper chromatography. The results given in Table I show that haemoglobin A_2 is different both from adult and fetal human haemoglobin[8, 14].

The presence of isoleucine is the most striking difference between haemoglobin A_2 and the main fraction, which lacks this amino acid[15]. It is interesting to note that ALLEN *et al.*[6] have observed the presence of isoleucine in the minor components they separated from human haemoglobin by chromatography on Amberlite IRC-50.

Haemoglobin A_2 is the chief minor component separated from crystallized haemoglobin by starch electrophoresis[4], and it is generally assumed not to exceed 2.5 % of the total pigment[4, 5, 16, 17]. Therefore the isoleucine content of haemoglobin A_2 (1.1 %) agrees well with the finding that isoleucine is not detectable by column chromatography[12] in crystallized preparations of adult-human haemoglobin[8, 18].

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Sporidesmolic acid B, a hydroxyacyldipeptide from *Sporidesmium bakeri*

Certain antibiotics[1-4] possess cyclic structures containing α-hydroxy- and α-amino acids linked by alternate ester and amide bonds. Alkaline degradation of these and similar[5] compounds gives hydroxyacylamino acids. We have isolated an hydroxyacyldipeptide from the fungus *Sporidesmium bakeri*, by saponification of a fraction which is believed to be neither toxic nor antibiotic.

Felts of sporing cultures, from which sporidesmin[6] had been removed, were dried from the frozen state and continuously extracted with methanol. The residue left after evaporation of the methanol was partitioned between the two phases of the system chloroform–methanol–water (10:7:3) and the material obtained by concentrating the lower phase extracted with ether. The insoluble residue was separated and extracted with chloroform; the extract was filtered, decolorized with charcoal and evaporated to dryness. The residue had m.p. 256–259° with prior sintering, and was obtained in a yield of 6.9 g/kg dry fungus. It was saponified in a large excess of methanolic NaOH. Removal of the methanol and acidification (HCl) gave a precipitate of "saponification acid"[7], about half of which dissolved in chloroform leaving a crystalline residue of sporidesmolic acid A. This possesses no free amino group, and yields on hydrolysis α-hydroxyisovaleric acid, valine, leucine and possibly N-methylvaline; it is probably a mixture.

The portion of "saponification acid" soluble in chloroform, termed sporidesmolic acid B, was obtained in a yield of 2.75 g/kg dry fungus. It crystallized from aqueous acetic acid as needles, m.p. 165–166° (corr.), $[\alpha]_D^{16°}$—108° in acetic acid (*c*, 4), readily soluble in organic solvents but sparingly soluble in water. A 1 % solution in aq. tetrahydrofuran had a pH of about 3; the acid dissolved in aq. $NaHCO_3$ and was precipitated unchanged on acidification. It produced no coloration with ninhydrin, migrated towards the anode on paper electrophoresis at pH 8 but was immobile at pH 2.5. The infrared spectrum showed peaks characteristic of amide, hydroxyl and carboxyl, but no absorption ascribable to ester carbonyl. (Found: C, 59.30; H, 9.35; N, 8.08. Calc. for $C_{17}H_{32}O_5N_2$: C, 59.24; H, 9.37; N, 8.13. Eq. wt. (by titration) 344, 347; calc.: 346.) Methyl ester (diazomethane), m.p. 105–106° (corr.). (Found: C, 60.16; H, 9.89; N, 7.55. Calc. for $C_{18}H_{34}O_5N_2$: C, 60.32; H, 9.56; N, 7.81. Mol. wt. (isopiestic, chloroform) 379; calc.: 359.)

Acid hydrolysis (6 *N* HCl, 110°, 72 h) furnished a mixture of α-hydroxyisovaleric acid, valine and N-methylleucine, each identified by paper chromatography in three or more different solvent systems using reference compounds as markers. Partial acid hydrolysis (conc. HCl, 37°) for 24 h liberated N-methylleucine and no other ninhydrin-positive substance. A trace of valine was present after 48 h. Ether extraction of a total acid hydrolysate and addition of cyclohexylamine to the ethereal extract gave cyclohexylammonium L-α-hydroxyisovalerate, $[\alpha]_D^{20°}$—9° in water (*c*, 4). An authentic sample had $[\alpha]_D^{20°}$—8.4°. The amino acids in the hydrolysate were separated by chromatography on cellulose powder in the system *tert*-butanol–aq. ammonia (s.g. 0.90)–water (20:1:4)[8]. N-methylleucine had $[\alpha]_D^{20°}$ + 20° in water (*c*, 1.3) (*cf.* ref.8 for L-isomer, + 20.4°); valine had $[\alpha]_D^{20°}$ + 47° in acetic acid (*c*, 2), (*cf.* ref.9 for L-isomer, + 62°). Although the valine was partly racemized, all the acids are clearly of the L-configuration. Sporidesmolic acid B is thus L-α-hydroxyisovaleryl-L-valyl-N-methyl-L-leucine.

S. bakeri is believed to be associated with facial eczema in sheep[10,11]: a powerful hepatotoxin, sporidesmin, has been isolated from cultures of the fungus[6]. A non-toxic metabolite with unusual properties has also been described[7]. Known for historical reasons as the "beaker test" substance, it may readily be isolated from felts of sporing cultures by the technique here outlined. Different samples have yielded on saponification up to 55 % of their weight of sporidesmolic acid B. Present evidence suggests that the "beaker test" substance is a mixture of related substances.

WHITE[7] has noted the similarity of the "beaker test" substance to the amidomycin group of antibiotics[1-4]. All have similar infrared spectra, contain hydroxy- and amino-acids, and differ from true peptides by their ease of saponification to yield acids with a terminal hydroxyacyl group. To describe such substances the term "peptolide" is suggested, the definition embracing derivatives of 2,5-dioxomorpholine such as the *Pseudomonas tabaci* toxin[5].

The "beaker test" substance differs from the peptolide antibiotics in possessing an α-hydroxyisovaleryl residue with the L-configuration, and in having a true dipeptide sequence. It may be significant that no antibiotic property has yet been shown for it. In fact, while direct evidence is lacking, its function may be that of a water-insoluble coat upon the fungal spores. The yield is greater from high-sporing than from low-sporing strains[12]. Electron micrographs of intact spores[13] show an apparently crystalline covering which is absent from spores which have been washed with organic solvents: the "beaker test" substance is known to be at least partly removed from dried fungal material by brief washing with acetone[14]. Experiments are planned to determine the effect of such treatment on spore viability, and to follow the appearance of the "beaker test" substance and of spores in growing cultures of *S. bakeri*. Biological examples are not wanting in which compounds of similar basic structure fulfil diverse roles, and peptolides may prove to be another example of such biochemical versatility.

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Isolation of thymidine diphosphate rhamnose and a novel thymidine diphosphate sugar compound from *Escherichia coli* strain B

Previous papers from this laboratory[1-4] presented evidence for the occurrence of new thymidine compounds in the bacteria, *Lactobacillus acidophilus* and *Escherichia coli*. The compound in *L. acidophilus* has recently been isolated and identified as thymidine diphosphate rhamnose (TDP-rhamnose)[4]. More recently the presence of three unidentified TDP-sugar compounds in a diaminopimelic acid-requiring mutant of *E. coli* has been shown by STROMINGER AND SCOTT[5]. The present paper reports the isolation of two TDP-sugar compounds from *E. coli* strain B. One of these compounds is identified as TDP-rhamnose.

If the growing cells of *E. coli* B were exposed to [^{3}H]thymidine, the labeled deoxyriboside was incorporated very rapidly into acid-soluble compounds and DNA, so that even after a few seconds exposure, the isotopic label was found in both the acid-soluble and DNA fractions. In one experiment cells exposed to [^{3}H]thymidine (3.0 μC/mμmole) for about 3 sec at 30° were chilled quickly to 0°, precipitated and washed. An acid-soluble extract was prepared from these cells and chromatographed on a Dowex 1 column with the formic acid system[6]. Besides the fractions of thymidine, TMP, TDP and TTP, a fraction eluted between TMP and TDP contained a large portion (44 %) of radioactivity.

For isolation of the compounds, the corresponding fraction was prepared as above in a large-scale experiment (performed with 19 l culture exposed to [^{3}H]-thymidine for about 10 sec) and subjected to paper chromatography in isobutyric acid-1 N NH_4OH (10:6). The paper chromatography resulted in separation of five ultraviolet-absorbing components. On examination of each component after elution from the paper, two major components (R_{TMP}, 0.66 and R_{TMP}, 0.86) were found to be radioactive and have the spectra of thymidine. Both contained two moles phosphate/mole thymidine and showed a reducing activity[7] equivalent to 1 mole rhamnose after mild hydrolysis with 0.01 N HCl at 100° for 10 min (Table I, TDP-X_1 representing the slower compound; TDP-X_2 the faster one). The microbiological assay after snake-venom treatment[1] of these compounds proved the presence of deoxyriboside in amounts expected from the ultraviolet absorption.

The cysteine-H_2SO_4 reaction for 6-deoxyhexoses (methylpentoses)[8] was positive with TDP-X_1 and gave approximately 1 mole 6-deoxyhexose/mole thymidine. The mobilities of TDP-X_1 and TDP-rhamnose isolated from *L. acidophilus* were identical

TABLE I

ANALYTICAL DATA OF TDP-X_1 (= TDP-RHAMNOSE) AND TDP-X_2

	Thymidine µmoles	*Total P µmoles*	*Reducing value* µmoles*	*6-Deoxyhexose µmoles*
TDP-X_1	1.00	2.04	0.99	0.92
TDP-X_2	1.00	2.00	1.00	0.18

* Expressed as rhamnose equivalent. The reducing value of rhamnose was about half that of glucose on a molar base.

Abbreviations: TMP, TDP, TTP, thymidine mono-, di- and triphosphate; DNA, deoxyribonucleic acid.

in isobutyric acid-NH_4OH as well as in ethanol-ammonium acetate. Furthermore, in four solvent systems tested, the R_F value of X_1 agreed with that of authentic rhamnose but differed from that of fucose (Table II). It leaves little doubt that TDP-X_1 is identical with TDP-rhamnose which was first isolated from *L. acidophilus*[4]*.

X_2 was chromatographed in pyridine–butanol–water (4:6:3) and located by $AgNO_3$ spraying. The spot could hardly be detected with aniline hydrogen phthalate which made visible X_1 as well as authentic rhamnose and fucose. The R_F value of X_2 was as high as that of rhamnose, suggesting that it might be a deoxyhexose.

TABLE II

R_F VALUES OF X_1 AND OF AUTHENTIC RHAMNOSE AND FUCOSE

Solvents	X_1	*Rhamnose*	*Fucose*
Butanol–acetic acid–water (4:1:5)	0.38	0.38	0.32
Butanol–ethanol–water (104:62:34)	0.48	0.48	0.42
Phenol saturated with water	0.49	0.49	0.56
Ethyl acetate–pyridine–water (8:2:1)	0.45	0.45	0.32

Diphenylamine[9], thiobarbituric acid[10] and *p*-nitrophenylhydrazine[11] reactions for 2-deoxysugars were negative. It failed to react with *p*-nitrophenylhydrazine even after periodate treatment, indicating that it is neither 3-deoxyhexose nor 3,6-dideoxyhexose[12]. In the cysteine-H_2SO_4 test for 6-deoxyhexoses, X_2 produced a reaction product with an absorption maximum at 400 mμ as did authentic rhamnose or fucose, but its extinction coefficient was as low as 20 % of that of these 6-deoxyhexoses. This fact might suggest that X_2 is somehow related to 6-deoxyhexose. The anthrone reaction[9] was positive, while all the following reactions[9] were negative: orcinol for pentoses and hexoses, cysteine-carbazole for ketosugars and trioses, carbazole-H_2SO_4 for uronic acids and Elson-Morgan for amino sugars.

Comparing the present results with those of STROMINGER AND SCOTT[5] it appears that none of the three TDP-sugars isolated by them is identical with either TDP-rhamnose or with TDP-X_2 reported here.

In the present experiment 8.5 μmoles TDP-rhamnose and 8.4 μmoles TDP-X_2 were obtained from 19 l culture. The specific activity of TDP-X_2 (31,720 counts/min/μmole) was much higher than that of TDP-rhamnose (9,480 counts/min/μmole). This fact may suggest more active turnover of the former within the cell.

Active incorporation of thymidine into the fraction containing TDP-rhamnose and TDP-X_2 is also observed in *E. coli* B 15 min after T2-phage infection[13]. Further studies are being made of the chemical nature and metabolic role of these TDP-sugar compounds.

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* However the possibility has not strictly been excluded that the sugar moiety of both compounds might be a stereoisomer of rhamnose.

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The presence of basic proteins in microsomes

The treatment of microsomes from rat liver with the detergents "Lubrol W" and sodium perfluoro-octanoate has revealed the presence of several protein fractions[1] into each of which amino acids are found to become incorporated to a different extent. Since the structure and composition of these fractions may throw light on their behaviour, in the course of the work the content of amino acids in the protein has been determined in several preparations of microsomes and their fractions. The N-terminal groups of the protein of some samples were also determined. Some amino acid analyses of the protein of whole microsomes[2] and of ribonucleoprotein particles[3] have previously been reported. Simkin and Work[4] gave the analyses of two fractions prepared by extraction of whole microsomes from guinea-pig liver with salt, which did not differ greatly from the whole microsome.

In the present work the protein of the microsomes of the Lubrol and PFO pellets were prepared as previously described[1]. The HCl extract was obtained by treating the PFO pellet with 0.2 N HCl for 16 h at 4°. The residue was re-extracted for 6 h, and the combined extracts dialysed and freeze-dried. The total amino acid analyses of the proteins were carried out by the FDNB method of Levy[5] and Fraenkel-Conrat, Harris and Levy[6] with the modifications described by Phillips and Johns[7]. The N-terminal groups were determined according to the technique of Phillips[8].

Table I gives the results of complete amino acid analyses of samples from three different preparations. The proportions of various N-terminal groups of some of the samples can be seen in Table II. The results show that the total protein of the microsomes is not predominantly basic since the sum of the acidic amino acids is greater than that of the basic. However, there is some tendency for the content of basic amino acids to increase in the Lubrol and PFO pellets. The HCl extract of the PFO pellet shows a marked increase of the basic residues indicating that there are among the microsomal proteins some which are markedly basic. The total percentage of basic amino acids in this fraction is 27.8, which is higher than that normally found in histones.

Abbreviations: PFO, perfluoro-octate; FDNB, fluorodinitrobenzene.

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As regards end-groups, it is remarkable to find that proline and alanine combined form almost 40 % (or even more) of the N-terminal groups both of the whole microsomes and of the Lubrol and PFO pellets. This may be compared with the results for histones of calf thymus in which these two N-terminal groups comprise at least 80 % of the total amount[7]. The main difference observed in the microsomes and their fractions is the high proportion of N-terminal glycine groups (25–33 %). As the latter may occur in a distinct protein, it is likely that the microsomes contain proteins similar

TABLE I

AMINO ACID COMPOSITION OF THE PROTEIN OF WHOLE MICROSOMES AND MICROSOMAL FRACTIONS

(moles % of amino acids determined)

Amino acid	*Whole microsomes I*	*Whole microsomes II*	*Lubrol pellet II*	*PFO pellet II*	*PFO pellet III*	*HCl extract of PFO pellet III*
Aspartic acid	8.8	10.1	9.1	8.0	8.3	7.5
Glutamic acid	12.7	11.5	10.7	11.2	10.6	10.4
Alanine	7.6	7.4	8.1	7.9	7.6	8.5
Valine	8.4	7.5	21.7	8.3	8.5	6.8
Leucine/isoleucine	13.9	15.7		14.9	13.7	11.7
Serine	6.3	6.1	6.6	6.3	5.2	5.3
Threonine	5.2	5.5	5.4	5.4	4.9	4.6
Glycine	7.3	6.8	7.5	7.4	9.4	7.5
Tyrosine	1.4	1.4	2.2	2.1	2.4	2.1
Phenylalanine	5.0	5.8	5.1	5.3	5.2	3.0
Methionine	1.0	—	0.7	—	—	—
Proline	5.9	5.9	5.6	5.4	4.7	4.8
Histidine	2.2	2.8	2.6	2.0	2.8	2.7
Lysine	6.6	6.8	8.0	8.4	8.1	13.6
Arginine	7.6	6.8	6.7	7.3	8.5	11.5

TABLE II

N-TERMINAL GROUPS OF THE PROTEIN OF WHOLE MICROSOMES AND MICROSOMAL FRACTIONS

(proportions of N-terminal groups expressed as percentages of the total end-groups found)

Amino acid	*Whole microsomes II*	*Lubrol pellet II*	*PFO pellet II*	*PFO pellet III*
Aspartic acid	10.2	3.1	3.2	3.0
Glutamic acid		2.7	2.6	2.6
Alanine	8.1	10.3	13.5	15.4
Valine	2.8	3.4	0.3	3.0
Leucine/isoleucine	2.8	2.0	2.4	2.1
Serine	10.0	8.0	5.0	8.1
Threonine	6.2	5.2	4.5	3.0
Glycine	25.1	31.1	33.1	28.7
Tyrosine	—	—	2.1	—
Phenylalanine	1.2	0.9	2.6	—
Proline	31.0	29.0	25.6	28.7
Histidine	—	—	0.5	—
Lysine	2.7	4.2	4.2	5.6
Arginine	—	—	—	—

in composition to the histones found in the nucleus. Further fractionations are being attempted.

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Announcement

5th Meeting of the International Conference on Biochemical Problems of Lipids

The 5th Meeting of the International Conference on Biochemical Problems of Lipids will take place in Marseilles (France) on July 21–23, 1960. Subject: "The enzymes of lipid metabolism". Sections: (1) Techniques, (2) Hydrolysis, (3) Oxidative degradation, (4) Biosynthesis.

Informations: Prof. P. Desnuelle, Faculté des Sciences, Place Victor Hugo, Marseilles (France).

SPECTRAL AND REDOX PROPERTIES OF BACTERIOCHLOROPHYLL IN ITS NATURAL STATE

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SUMMARY

The behaviour of bacteriochlorophyll in its natural state with regard to oxidative bleaching, photobleaching and temperature bleaching was investigated. Reversible changes in absorption by the addition of oxidative and reductive agents, previously measured in organic solution, were also found to be present when bacteriochlorophyll is in its natural state in chromatophores of purple bacteria. Only the absorption band of longest wavelength in *Rhodospirillum rubrum*, *Rhodopseudomonas spheroides* and *Chromatium* could be bleached reversibly. Also a reversible shift in position of the 800-mμ absorption band in *Rh. rubrum* occurs while there are indications of a similar shift in the other species. At a potential of approximately 515 mV the difference spectrum obtained by addition of ferri-ferrocyanide mixtures to chromatophores of *Rh. rubrum* was similar to the difference spectrum obtained by Duysens by illumination of living bacteria. Additional bleaching experiments were done by heating and illumination of the chromatophore extracts.

The present experiments may yield a link between the *in vitro* and *in vivo* phenomena obtained with chlorophyllous pigments.

INTRODUCTION

The study of the chemistry and photochemistry of the chlorophylls has been confined predominantly to pigments extracted from the cells and dissolved in organic solvents. In the photosynthetic apparatus these pigments occur under conditions quite different from those in organic solution. For example, the pigment concentration in the photosynthetic structure usually is more than a thousand times higher than the concentration used in most experiments with chlorophylls in organic solvents. Also there are many indications that the pigments cannot move freely in the structure as they do in organic solvents, but are concentrated in thin layers and probably attached to carriers such as protein macromolecules[1]. These differences are reflected, among other ways, in differences in both absorption spectra and fluorescence yields.

It seems promising to investigate which of the *in vitro* properties of the chlorophylls are retained when the pigments are present in their natural state and how these properties are modified under these conditions.

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For this type of investigation bacteriochlorophyll proved to be the most useful pigment. There is a considerable difference in absorption spectra of bacteriochlorophyll dissolved in organic solvents and bacteriochlorophyll located in photosynthetic bacteria. Moreover, rather stable extracts of chromatophores can be prepared from these bacteria. These extracts usually have the same absorption spectrum as living bacteria.

Earlier experiments made with bacteriochlorophyll in organic solution[1,2] showed that this pigment could be bleached by addition of different oxidants, such as ferric chloride, iodine or potassium permanganate. The original spectrum could be restored completely by immediate addition of ferrous salts, ascorbic acid or other reductants. In contrast to the behaviour of chlorophyll *a*[2], no slow restoration of the spectrum occurred upon standing in the dark or addition of non-reducing salts. With bacteriochlorophyll—as well as with chlorophylls *a* and *b* and bacterioviridin—dissolved in methanol it was possible to measure a fairly reproducible oxidation-reduction potential[3].

The "oxy" chlorophyll forms are unstable in air saturated solution. They lead to different secondary products in the case of bacteriochlorophyll and chlorophyll *a*.

We investigated the capacity for reversible oxidation-reduction of bacteriochlorophyll in its natural state in the different modifications occurring in various photosynthetic purple bacteria, and also the question was considered whether this redox phenomenon can be related to absorption changes in the bacteriochlorophyll spectrum measured by DUYSENS[4] with living bacteria. In connection with these phenomena the absorption changes brought about by heating and intense illumination were also studied.

METHODS

Chromatophores of the photosynthetic purple bacteria *Rhodospirillum rubrum* strain 1-1-1, *Rhodopseudomonas spheroides* and *Chromatium* strain D were obtained by the use of a FRENCH-MILNER homogeniser[5]. Intact bacteria and cell debris were removed by 10-min centrifugation at 5000 × *g*. The chromatophores then were precipitated by 1-h centrifugation at 10,000 × *g* and the supernatant discarded. The pellet was taken up in distilled water or a buffer solution. As a rule phosphate buffer pH 7.5 was used.

Absorption spectra and difference spectra were measured with a Beckmann DK-2 recording spectrophotometer. Difference spectra were obtained by addition of the required amount of a ferri-ferrocyanide mixture to one of the cuvettes with bacterial extract, while an equal amount of distilled water was added to the other cuvette. A correction was applied for the absorption of the ferri-ferrocyanide mixture. Experiments on chromatophore bleaching were carried out by illumination with a 500 or 1000 W incandescent lamp with light absorbed only by bacteriochlorophyll which was isolated by a Corning 3480 filter and a 5-cm water filter.

For the determination of the shift in position of the 800-mμ absorption band, occurring when living bacteria and chromatophores are illuminated, the derivative spectrophotometer of FRENCH[6] was used.

RESULTS

The oxidation-reduction potential of bacteriochlorophyll in methanol (the potential of the solution at which the infrared absorption band with a maximum at 770 mμ is bleached halfway) was previously found to be approximately 550 mV *versus* a standard hydrogen electrode. This value suggested that reversible oxidation-reductions of aqueous chromatophore suspensions might be measured by the use of ferri-ferrocyanide systems. These systems also have the advantage that it is possible to work with solutions buffered at pH 7.5. Thus absorption changes and coagulation due to low pH as well as the influence of low and probably non-physiological pH on potential values are eliminated (*cf.* THOMAS *et al.*[7]).

The results of absorption and potential measurements obtained with chromatophores of three different bacterial species, each having a different near infrared absorption spectrum due to bacteriochlorophyll, will be given. Two of these species belong to the group of non-sulfur bacteria, the third to that of the sulfur bacteria.

Rhodospirillum rubrum *strain 1-1-1*

Fig. 1a shows the near infrared absorption spectrum of a chromatophore suspension from *Rhodospirillum rubrum* and Fig. 1b the absorption spectrum of a methanolic bacteriochlorophyll solution. In this bacterium both the main infrared absorption band at 880 mμ and the weak absorption band at about 800 mμ in the natural state correspond to the 770-mμ absorption band of bacteriochlorophyll in methanol[2]. Thus the main fraction of the pigment molecules shows a greater shift in absorption maximum—from the *in vitro* position—than a minor fraction of the pigment molecules. It can be estimated that at 800 mμ the absorption due to the

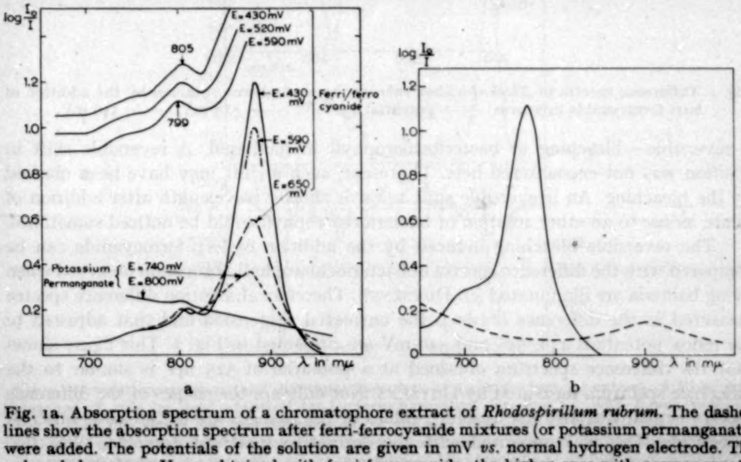


Fig. 1a. Absorption spectrum of a chromatophore extract of *Rhodospirillum rubrum*. The dashed lines show the absorption spectrum after ferri-ferrocyanide mixtures (or potassium permanganate) were added. The potentials of the solution are given in mV *vs.* normal hydrogen electrode. The values below 700 mV are obtained with ferri-ferrocyanide, the higher ones with permanganate. In the insert the position of the 800-mμ band is given at three different potentials. 1b. Near infrared part of the absorption spectrum of a methanolic extract of *Rhodospirillum rubrum*. The dashed lines give the spectrum after addition of ferric chloride, iodine or potassiumpermanganate.

molecules with the small shift is approximately one third of the total absorption at this wavelength. Then the number of molecules showing a shift to 800 mμ is of the order of 1 % of those showing a shift to 880 mμ.

The results of addition of ferri-ferrocyanide mixtures with different potential and of potassium permanganate are also presented in Fig. 1a. It appears from this figure that with increasing potential two different processes occur: the 880-mμ band is bleached while the 800-mμ band is shifted in position from 805 to 799 mμ but not bleached. Even after a total bleaching of the 880-mμ band the weak band is still visible at 799 mμ. The potential at which the 800-mμ band is halfway shifted is approximately 520 mV, while the potential at which the 880-mμ band is halfway bleached is approximately 650 mV. The bleaching of the 880-mμ band is not fully reversible: the sooner the addition of reductant the higher the percentage of reversibility. This is similar to the behaviour in organic solvents, where complete reversibility is obtained only after immediate addition of reductant. Fig. 1b shows the —reversible—bleaching of bacteriochlorophyll in methanol. A reversible shift in position was not encountered here. If present, such a shift may have been masked by the bleaching. An irreversible shift towards shorter wavelength after addition of dilute iodine to an ether solution of bacteriochlorophyll could be noticed sometimes.

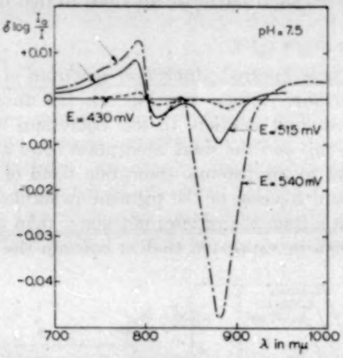


Fig. 2. Difference spectra of *Rhodospirillum rubrum* chromatophores obtained by the addition of ferri-ferrocyanide mixtures: ---- potential 430 mV, —— 515 mV, —·— 540 mV.

The reversible bleaching induced by the addition of ferri-ferrocyanide can be compared with the difference spectra of bacteriochlorophyll absorption observed when living bacteria are illuminated (*cf.* DUYSENS[4]). Therefore absorption difference spectra measured as the difference between the untreated suspension and that adjusted to the redox potentials 430, 515 and 540 mV are presented in Fig. 2. This figure shows that the difference spectrum obtained at a potential of 515 mV is similar to the difference spectrum measured by DUYSENS. Not only are the shapes of the difference spectra similar but also the absolute values of the changes are of the same order of magnitude. This was measured with the derivative spectrophotometer. With this instrument the determination of the exact location of the maximum of the 800-mμ band is possible. Side illumination with blue light (isolated by a Corning 5031 filter and a 5-cm 6 % copper sulfate solution) of $1.1 \cdot 10^4$ ergs/cm^2/sec produced a band shift from 805 to 800 mμ with bacteria suspended in distilled water. This shift was reversed after a few seconds storage in the dark. When the intensity was reduced to a tenth, the shift still was 3 mμ, suggesting that the higher intensity was sufficient to cause the maximum possible shift. In chromatophore suspensions the reversible shift upon illumination was only 1 or 2 mμ, while addition of ferricyanide in the dark to the same extract caused a shift of 5 mμ.

A chromatophore suspension is bleached irreversible if illuminated with strong light (approximately 150,000 f.c. with filters removed). The stability against bleaching of bacteriochlorophyll in chromatophores exceeds the stability for bacteriochlorophyll dissolved in methanol more than a thousand times. Irreversible photobleaching was found to proceed in freshly prepared extracts at a rate approximately 4 times higher than in extracts which had been stored in the dark for a considerable time. Usually these freshly prepared extracts also showed photophosphorylation. Another chemical activity exhibited by these freshly prepared extracts is their capacity to reduce ferricyanide in the dark after addition of succinate or growth medium (peptone). The reduction of ferricyanide in the dark could be used to demonstrate the reversibility of the oxidative bleaching with chromatophore suspensions: addition of an equal amount of succinate to the cuvettes in the spectrophotometer, both containing chromatophores, one of them with ferricyanide, resulted in a gradual disappearence of the difference spectra presented in Fig. 2.

Photobleaching at these high light intensities usually was not totally irreversible. Up to 10 %, and in some cases up to 30 %, of the bleaching proved to be reversible. As shown in Fig. 3, the difference spectrum in the near-infrared also consists of a decrease of the 880-mμ band and a shift of the 800-mμ band. This spectrum differs

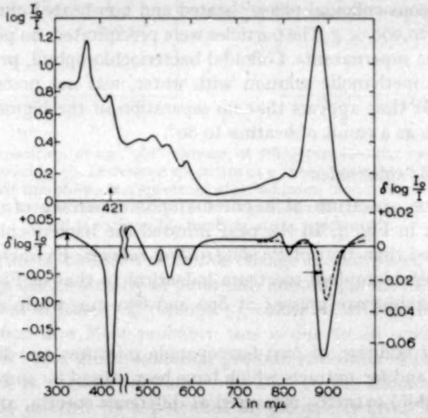


Fig. 3. Absorption spectrum and difference spectrum of a chromatophore extract of *Rh. rubrum* bleached 15 min by light absorbed by bacteriochlorophyll (150,000 f.c. with filters removed). The dashed line shows the near infrared part of the spectrum after 15 min storage in the dark, in the rest of the spectrum no measurable change in absorption occurred during this dark period. The marked decrease in absorption at 421 mμ is due to the bleaching of un unknown ether soluble pigment, present to an unusually large extent in this special extract. Whether the increase around 340 mμ (marked by left arrow) is due to this same pigment or to a different one, could not be established.

from the completely reversible difference spectrum, measured by DUYSENS at much lower intensities of illumination, in the ratio between the values of decrease and shift. Bacteriochlorophyll bleaching in organic solvents also is slightly reversible. After a nearly complete bleaching of the 770-mμ band by light this band regenerated about 10 %. The percentage of reversibility could be enhanced to 70 % by addition of ascorbic acid, but only in alcoholic solvents. With chromatophores no increase in the percentage of reversibility of photobleaching of bacteriochlorophyll was found after addition of ascorbic acid.

Fig. 3 also shows irreversible decrease in absorption (occurring after a few minutes of illumination) in the short wavelength part of the spectrum (421 mμ). This decrease was due to an ether soluble pigment different from bacteriochlorophyll, carotenoids and most probably also from cytochromes[8].

The temperature of the chromatophore extract rose from 3 to 5° during 15 min illumination with strong light. Therefore it was investigated whether heating of the extract exhibits a marked influence on the absorption spectrum. Heating the extract from room temperature (20°) to 30° or cooling it 10° resulted in the appearance of a difference spectrum of approximately the same shape as measured with high intensity photobleaching. The magnitudes of these differences were smaller than those encountered in the bleaching experiments. It thus seems unlikely that the high intensity reversible photobleaching is due to the influence of temperature only. After heating a freshly prepared extract for less than 1 min at 80°, the absorption band at 880 mμ disappears while a new band arises st about 780 mμ, in a position close to that of bacteriochlorophyll in organic solvents. In order to investigate whether or not bacteriochlorophyll in this state is separated from the chromatophores and present in an aqueous colloidal phase, heated and non-heated chromatophores were centrifuged 1 h at 20,000 × *g*. The particles were precipitated, no pigment was present in either one of the supernatants. Colloidal bacteriochlorophyll, prepared by dilution of a concentrated methanolic solution with water, was not precipitated under the same conditions. It thus appears that no separation of the pigment from the chromatophores occurs as a result of heating to 80°.

Rhodopseudomonas spheroides

The absorption spectrum of a chromatophore extract of *Rhodopseudomonas spheroides* is given in Fig. 4. In the near infrared the bacteriochlorophyll spectrum is more complicated than that of *Rhodospirillum rubrum*. Extraction with methanol results in a bacteriochlorophyll spectrum indentical to that of Fig. 1a. In the chromatophore two maxima are present at 800 and 850 mμ, while a shoulder appears at about 890 mμ.

The results of addition of ferri-ferrocyanide mixtures are different for freshly prepared extracts and for extracts which have been stored for some time. The results with the latter ("old") extracts, measured as difference spectra, are given in Fig. 5a. A reversible decrease in absorption occurs at 885 mμ and around 805 mμ, a reversible increase around 790 mμ. This difference spectrum looks similar to that measured with *Rhodospirillum rubrum*. Also at potentials between 600 and 650 mV only the long wave length shoulder in the absorption spectrum appears to be bleached reversibly. At potentials higher than 700 mV a marked irreversible decrease occurs with the other bands.

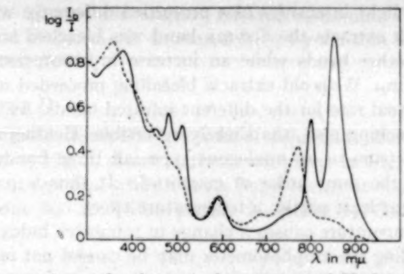
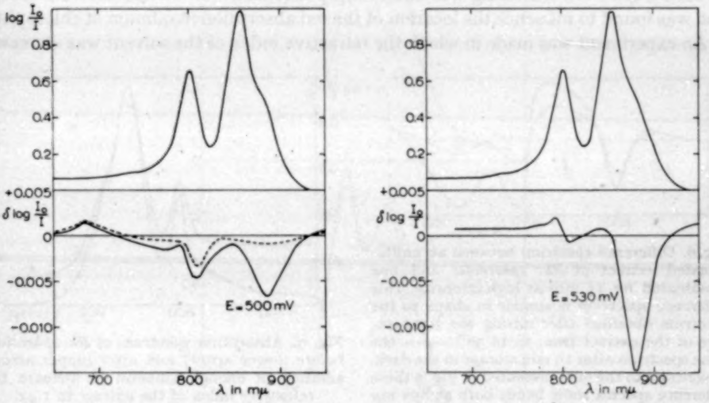


Fig. 4. Absorption spectrum of a chromatophore extract of *Rhodopseudomonas spheroides* before (——) and after (----) a 1-min heating to 83°.



a b

Fig. 5a. Difference spectrum of an "old" extract of *Rhodopseudomonas spheroides*, obtained by the addition of ferricyanide. 5b. Difference spectrum of a "fresh" extract of *Rh. spheroides* buffered at pH 7.5. The dashed line shows the spectrum after addition of succinate to the cuvettes. Such an addition results in a reduction of ferri- to ferrocyanide and consequently in a disappearence of the reversible fraction of the spectrum.

With fresh extracts, besides a reversible change in absorption, also an irreversible decrease at 800 mμ occurs already at potentials around 500 mV. This decrease, which is strongly enhanced at lower pH (around 5), coincides with an irreversible increase in absorption at 690 mμ. Most probably this is due to an oxidation of "800 mμ bacteriochlorophyll" to a green oxidation product encountered in experiments with bacteriochlorophyll in organic solution[2,9]. In Fig. 5b the difference spectrum with a ferri-ferrocyanide mixture at a potential of 500 mV is given for a fresh extract buffered at pH 7.5. In this figure the reversible and irreversible fractions are given by the curve obtained after addition of succinate to the oxidised chromatophore extract, as such an addition results in a reduction of ferricyanide and a disappearence of the reversible bleaching. The graphs indicate that the reversible change in absorption in this spectral region most probably is similar to that measured with "old" extracts.

Bleaching at high light intensities also proceeded differently with "fresh" and "old" extracts. In fresh extracts the 800-mμ band was bleached at an appreciably higher rate than the other bands while an increase in absorption occurs with a maximum around 690 mμ. With old extracts bleaching proceeded much slower and at an approximately equal rate for the different infrared bands. As shown in Fig. 6, this high intensity bleaching also was slightly reversible. Heating and cooling produced a difference spectrum of an analogous type (all three bands are present in this spectrum) and of the same order of magnitude. It thus is possible that this reversible bleaching is, at least partly, a temperature effect.

As a change in temperature causes a change in refractive index, the differences measured in the recording spectrophotometer may be caused not only by a change in absorption, but also by a change in selective scattering of the extract. The existence of such a selective scattering was observed by LATIMER[10] in chloroplast suspensions and was found to influence the location of the red absorption maximum of chlorophyll *a*. An experiment was made in which the refractive index of the solvent was increased to 1.42 by addition of albumin. As shown in Fig. 7, the scattering is increased, but the location of the absorption maxima is not influenced by this treatment. Also the difference spectrum upon heating was not influenced as should be expected if this difference spectrum were due to selective scattering. It thus seems doubtful that the temperature effects are due to scattering and not to absorption differences. The results of a 1-min heating at 80° of a chromatophore extract of *Rhodopseudomonas spheroides* is also presented in Fig. 4. The near infrared absorption spectrum of the extract after heating looks similar to the spectrum of bacteriochlorophyll dissolved in organic solvents, shown in Fig. 1b. Centrifugation experiments showed that, analogous to the results with *Rhodospirillum rubrum* extracts, the pigment had not been released from the chromatophores, but it may be released from its normal position in the chromatophores. The remnants of the carotenoid bands and the bacteriochlorophyll band in the near u.v. also are shifted towards shorter wavelengths. No fluorescence could be measured with these heated extracts. The new band at 780 mμ, however, is broader than the bands of the original spectrum and the corresponding band in organic solvents. The area covered by this band is approximately

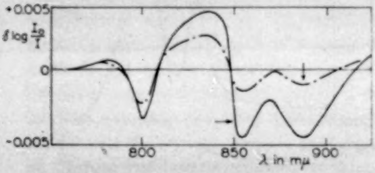


Fig. 6. Difference spectrum between an unilluminated extract of *Rh. spheroides* and one illuminated for 15 min at high intensity. This difference spectrum is similar in shape to the spectrum obtained after raising the temperature of the extract from 20 to 30°. —·— the same spectrum after 10 min storage in the dark. In contrast to the ones presented in Fig. 5 these difference spectra show bands both at 850 mμ and 890 mμ as indicated by arrows.

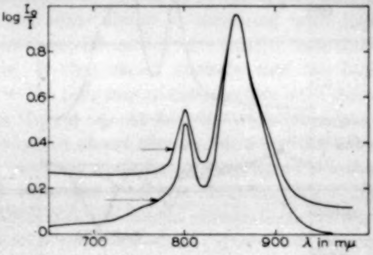


Fig. 7. Absorption spectrum of *Rh. spheroides* before (lower arrow) and after (upper arrow) addition of enough albumin to increase the refractive index of the extract to 1.42.

one third of the area of the original infrared spectrum. The height of the most pronounced band at 850 mμ is decreased 80 % by a 1-min heating to 76° and 20 % by a 1-min heating at 60°.

Chromatium *strain D*

The near infrared absorption spectrum of a chromatophore extract of *Chromatium* strain D as given in Fig. 8, shows marked variability. In Fig. 8a the main absorption band is located at 850 mμ, while a lower band occurs at 800 mμ and an absorption shoulder at about 890 mμ. This picture is similar to that of *Rhodopseudomonas spheroides*, although the half widths of the bands of the latter species are markedly smaller. In the absorption spectrum presented in Fig. 8b the main band is located at 800 mμ. In some cases the band at 850 mμ is even much less pronounced. Although it is difficult to measure the total absorption due to a band responsible for the absorption shoulder at 890 mμ, it is estimated that the absorption due to this band is of the same order of magnitude in both types of spectra. The remark of WASSINK

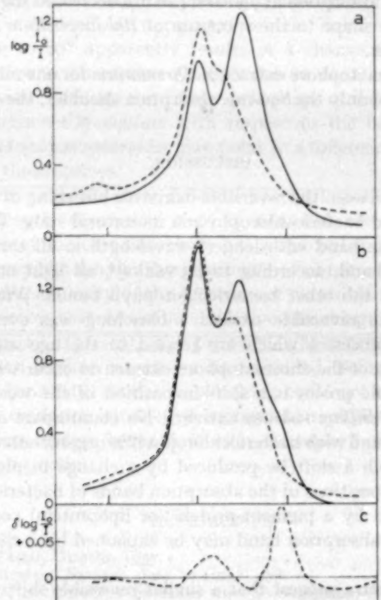


Fig. 8a and 8b. Near infrared absorption spectra of extracts of *Chromatium* strain D. The effect of addition of ferri-ferrocyanide mixtures is indicated by a dashed line. 8c. Difference spectrum drawn from the values obtained when reducing agent (ascorbic acid) was added to the extract of Fig. 8b.

et al.[11] that both types are interconvertable by a change of light intensity during the period of growing could not be confirmed as yet. A decrease of the light intensity from 1000 to 200 lux had no effect on the absorption spectrum.

Addition of ferri-ferrocyanide resulted in an irreversible decrease in absorption of the 850-mμ band, more so in freshly prepared extracts. In some cases an irreversible increase of the 800-mμ band was observed. Reversible bleaching was found to occur only with the 890-mμ absorption shoulder. Fig. 8c shows the difference spectrum obtained after addition of ascorbic acid as reductant of the oxidised chromatophore extract. This addition resulted in an increase in absorption at 890 mμ and 815 mμ, while the absorption at 780 and 820 mμ decreased slightly. This difference spectrum is similar in shape to the spectrum of *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides*.

Heating of a chromatophore extract of *Chromatium* for one minute at 83° resulted in the disappearance of only the 890-mμ absorption shoulder, the other bands seemed to be unaffected.

DISCUSSION

There is a similarity between the reversible oxidative bleaching of bacteriochlorophyll in organic solvents and bacteriochlorophyll in its natural state. This similarity holds only for the absorption band with longest wavelength in all three bacterial species investigated. To this band, according to DUYSENS[12], all light energy is transferred which is absorbed by the other bacteriochlorophyll bands. With these other near infrared absorption no reversible oxidative bleaching was evident. However, irreversible bleaching processes which are related to the age and probably to the photochemical activity of the chromatophore extract do occur with these bands.

A second reversible proces is a shift in position of the weak absorption band at 800 mμ in *Rhodospirillum rubrum* extracts. No counterpart of such a reversible shift in position was found with bacteriochlorophyll in organic solvents. It is, however, not necessary that such a shift be produced by a change in pigment composition. If we assume that the positions of the absorption bands of bacteriochlorophyll *in vivo* are mainly determined by a pigment-protein (or lipoprotein) combination, a small shift in position of an absorption band may be explained by a change in the protein part as well.

The difference spectra suggest that a similar reversible shift occurs with *Rhodopseudomonas spheroides* and *Chromatium* strain D, although here it concerns only a small fraction of the total absorption at 800 mμ. Consequently in these latter species an absorption spectrum similar to that of *Rhodospirillum rubrum*—showing reversible oxidative changes—seems to underlie the total absorption spectrum. It may be a plausible hypothesis that the spectrum as presented by *Rhodospirillum rubrum* is a basic spectrum for the functioning of photosynthetic purple bacteria. In this respect it may also be mentioned here that this latter spectrum appears, according to SISTROM *et al.*[13] in carotenoid free photosynthetic mutants of *Rhodopseudomonas spheroides*.

The difference spectrum obtained by illumination of *Chromatium* (*cf.* DUYSENS[4]) showing a reversible decrease at 890 and 800 mμ and a reversible increase at about 790 mμ, may also be explained in terms of reversible bleaching of the 890-mμ shoulder and a shift of a small fraction of the 800-mμ band.

The two phenomena of bleaching and shift may be caused either by a single process or by two different processes. The finding that the shift of the 800-mμ absorption band upon illumination is smaller with chromatophores than with living bacteria, while DUYSENS mentions that the bleaching of the 890-mμ band with chromatophores exceeds the bleaching with bacteria, might point to the latter of the two possibilities. In this respect also the relatively low value of light saturation of the shift may be mentioned.

The changes of the near infrared absorption spectrum upon heating to 80°, especially with *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides*, is in accordance with the hypothesis that the position of the bacteriochlorophyll absorption bands is mainly determined by a pigment–protein linkage. Denaturation of the protein by heating to 80° apparently results in a change to the *in vitro* type of spectrum combined with absorption changes due to denaturation of the pigment. The different behaviour and also the difference in shape of the 800- and 850-mμ bands of the sulfur bacterium *Chromatium* with respect to the bands of the non-sulfur bacteria *Rhodopseudomonas spheroides* may point to a difference in molecular pigment structure between those species.

The experiments with the three different bacterial species, indicating that only the longest wavelength absorption band is bleached reversibly, show that the molecular structure of the chromatophore has a marked influence on the chemical properties of bacteriochlorophyll. The possibility of obtaining with chromatophores at a given potential a similarity of the spectrum of reversible bleaching by light with living bacteria indicates that reversible oxidative bleaching can play a role *in vivo*. Whether this property represents the primary light reaction of bacterial photosynthesis or merely a sideline cannot yet be concluded. Being specific to the chlorophyllous pigments (reversible oxidative bleaching could not be observed with pheophytins, the green oxidation product of bacteriochlorophyll or with carotenoids) it gives one of the first bridges between *in vitro* chlorophyll chemistry and processes occurring in the living cell.

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THE PHYSICAL STRUCTURE OF WHEAT PROTEIN

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SUMMARY

The physical structure of wheat proteins has been investigated using both wide- and small-angle X-ray scattering and electron microscopy. Dry wheat gluten, gliadin and the water-soluble proteins all give similar wide-angle diffraction patterns characteristic of folded polypeptide chains in the α_1-helix configuration. Small-angle scattering curves from stretched, dried glutens show marked orientation and can be interpreted in terms of parallel sheets of gluten platelets whose thickness does not exceed ~70 Å. Electron micrographs taken of surface replicas of the stretched glutens confirm the sheet structure. Moist, relaxed glutens, while not exhibiting the orientation into sheets, still give the X-ray scattering characteristic of platelets.

Several small-angle diffraction peaks observable in both dry and moist glutens can be traced to the bound lipids which are extractable with water-saturated *n*-butanol. These peaks correspond to d-spacings of 62 Å and 48 Å. The extracted lipid exhibits the same structure and suggests that gluten originally consists of a lipoprotein complex. Removal of the bound lipid deprives the gluten complex of its elastic properties and it is not possible to obtain oriented small-angle X-ray patterns from stretched samples, although the platelet-scattering persists isotropically. Electron micrographs confirm the absence of sheet structure in lipid-free glutens. Gliadin films, prepared by evaporation of the ethanol extract exhibit a strong 42 Å diffraction which can be removed by extracting the bound lipid. The extracted lipid, however, exhibits its strongest diffraction at 48 Å, indicating that the lipo–gliadin complex is slightly skewed or compressed.

INTRODUCTION

The investigation of the microphysical structure of wheat protein has been largely confined to the work of HESS[1], and TRAUB, HUTCHINSON AND DANIELS[2], who studied the structure by means of X-ray diffraction and electron microscopy.

HESS showed that the native protein in the wheat grain could be physically separated into wedge (between starch grains) protein and bound (to the starch grains) protein. Two X-ray diffractions were obtained from these fractions, one representing a spacing d ≈ 45–47 Å and the other d ≈ 80-90 Å. TRAUB later showed that this latter spacing was due to starch. All spacings are observed to increase slightly with hydration of the flour. Dry glutens washed from flour doughs were also observed to give the ~47 Å line. TRAUB succeeded in showing that this line was in reality due to the phospholipid bound in wheat protein. Because of the disruptive effect on the elasticity of wheat doughs occasioned by the removal of the bound lipids[3], both HESS and TRAUB concluded that the lipid was intimately connected with the protein structure. HESS pictured the lipids forming layers between the protein fibrils and entering strongly into the hydration reaction; TRAUB suggests that the protein fibers are held together by layers of phospholipids in the form of bimolecular leaflets.

The present work seeks to elucidate further the wheat protein structure and the role of lipids in that structure by a combined X-ray and electron microscope approach. For the most part the earlier work of HESS and TRAUB is confirmed and some new results are reported.

EXPERIMENTAL

A. *Preparation of samples*

All protein samples were prepared from unbleached, untreated flours. Two were pure strains. Table I gives the data pertinent to the flours*.

TABLE I

Flour No.	*Description*	*% Protein*
434	Montana Hard Spring Wheat	13.8
3237	Pawnee (pure strain, short dough development time)	12.8
3238	C11Z871 (pure strain, long dough development time)	12.8

Glutens were washed from flour-water doughs in the usual way with distilled water and lyophilized to obtain dry samples. Lipid extraction was performed following the method of MECHAM AND MOHAMMAD[3], using water-saturated *n*-butanol. Gliadins were extracted from the dry glutens using 70 % ethanol. Water-soluble protein was obtained using the procedure of MATTERN AND SANDSTEDT[4].

Birefringence studies on moist, stretched gluten[5] have shown that relaxation is 90 % complete in ~1 ½ h. Since most X-ray exposures and the preparation of electron microscope samples require at least this length of time, observation of the microstructure cannot be made unless relaxation is prevented. This has been achieved by first stretching the gluten unidirectionally as a thin sheet over a smooth metal button held at ~ —70°. The gluten freezes within a few seconds and is then transferred quickly to a small vacuum enclosure where it is dried by lyophilization. The dried sample, white in color, retains the size and shape of the original stretched specimen even to the oriented strain markings on the surface.

B. *X-ray apparatus*

Conventional wide-angle X-ray diffraction patterns were taken with a Laue camera using Cu-Kα radiation. Small-angle patterns were taken using either (1) the Laue camera with a film-specimen distance of 180 mm, or (2) a specially constructed small-angle camera using proportional counter detection of the X-rays.

The special small-angle camera was of the 4-slit type[6] and has been described elsewhere[7]. The collimating system is evacuated to forepump pressure ($\sim 1.0\ \mu$) to reduce air scattering; however, the sample is at atmospheric pressure. "Mylar" (0.00025 in.) windows[8] are used at the entrances and exits of the vacuum system.

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* Flour 434 supplied by Pillsbury Mills. Flours 3237 and 3238 supplied by Kansas State University.

Monochromatization of the X-rays is achieved electronically using the characteristics of the proportional counter in conjunction with a single-channel pulse-height analyzer (Atomic Model 510). Copper Kβ is suppressed with a Ni filter.

C. *Electron microscope techniques*

An RCA EMU-2 electron microscope modified with a compensable pole-piece and Canalco astigmator was used in these studies. Resolution of the order of 40 Å could be obtained when the microscope column was clean and well aligned.

Wheat proteins do not lend themselves easily to direct observation under an electron beam; however, a solution of the difficulties is the use of surface replicas. Freeze-dried, stretched samples, prepared in the manner described above, were first shadowed in vacuum with Pt-Pd alloy (80–20%) at a glancing angle of 14 degrees. Carbon was then evaporated normally onto the surface. The sample was then removed from the evaporator and pressed, under heat ($\sim$100°), replica down, into a piece of polystyrene sheet. This "sandwich" was washed alternately in 70% ethanol and 0.2% KOH to remove the gluten. Following washing, the sandwich (open-face) was submerged in ethylene dichloride which dissolved away the polystyrene leaving the shadowed carbon replica floating freely. The main advantage of this procedure is that the replica is made prior to any of the heat treatment or washing procedures and hence records faithfully the original state of the gluten surface.

RESULTS AND DISCUSSION

A. *X-ray*

1. *Wide-angle diffraction*. The proteins extracted from wheat are not crystalline in nature and should therefore not be expected to give a sharp wide-angle diffraction pattern. Fig. 1(a) is a typical diffraction pattern taken of dry, powdered wheat gluten. The patterns from dry gliadin, glutenin and the water-soluble wheat proteins are all essentially the same.

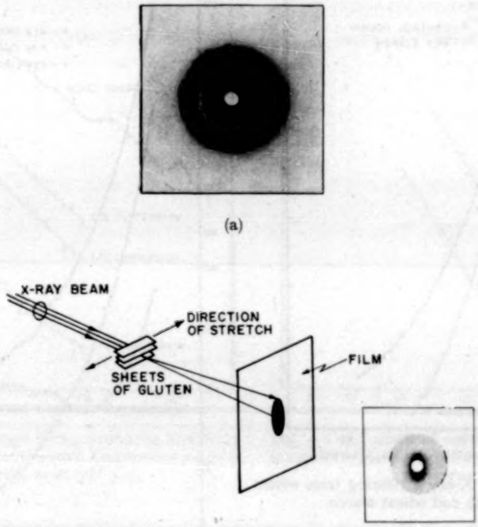
ARNDT AND RILEY[9] have shown that the patterns obtained from a wide range of animal proteins fall into three distinct classes which they term α, β, γ. The first group contains the largest number and consists of such corpuscular proteins as egg albumin, ribonuclease, and hemoglobin. The patterns for wheat proteins most nearly agree with the α-group proposed by RILEY AND ARNDT, which, according to these authors, consists of folded polypeptide chains in the α_1-helix configuration of PAULING AND CORY[10].

2. *Small-angle scattering*. Because of possible confusion introduced by extraneous components of the wheat (*i.e.*, starch, lipids, etc.), it is desirable first to isolate the contribution of these components to the small-angle scattering.

(a) *Doughs*. The structure of wheat protein is of interest here because it is related to the behavior of flour doughs used in bread making. One would therefore wish to observe this structure directly as it exists in a developed dough.

Fig. 2 shows the small-angle scattering from a straight flour–water dough and from the wet starch extracted from the dough. It is clear that starch, which comprises

$\sim$85 % of the dough, is responsible for the major shape of the scattering from dough, and that the protein must be extracted and studied separately if much is to be learned of its structure. The peak at $\sim$12 milliradians seen in Fig. 2 corresponds to a Bragg d-spacing of $\sim$125 Å. HESS[1] first ascribed this interference to the protein but TRAUB[2] has since shown it to arise from starch as is demonstrated in the present work.



(a)

(b)

Fig. 1(a). Wide-angle X-ray diffraction from amorphous gluten. Film-specimen separation, 4 cm.
Fig. 1(b). Small-angle X-ray scattering from stretched, dry glutens showing orientation effects. Film-specimen separation, 18 cm.

Whether the maximum is a true Bragg reflection or is due to interparticle interferences within the starch is not clear from any of the work done to date. It does not, however, appear pertinent to the structure of wheat protein, either isolated or within a parent dough.

(b) *Glutens*. The small-angle scattering from dry powdered gluten is shown as the uppermost curve in Fig. 3. The undulations observed in the angular range 20–40 mr. are reproducible and due to the lipid component ($\sim$7 % by weight). The scattering from the extracted lipid and the gluten residue are shown displaced beneath the parent gluten curve. It is usually not possible to remove completely the lipid lines from the gluten residue.

The interferences from the lipid are thought to be true Bragg diffractions with d-spacings 62 Å and 48 Å. HESS[1] assigned these lines to the protein, although he did report their disappearance from glutens washed from defatted flours. It is significant that (1) the removal of lipids does not seriously alter the general shape of the gluten scattering curve, and (2) the positions of the diffraction lines from the lipid do not change from the bound to the isolated state, although their sharpness in the isolated state is strongly dependent on the degree of solidification of the sample. These observations would indicate that the lipid, while bound into a lipoprotein complex in the gluten, separates essentially as a unit under the solvent action of wet *n*-butanol.

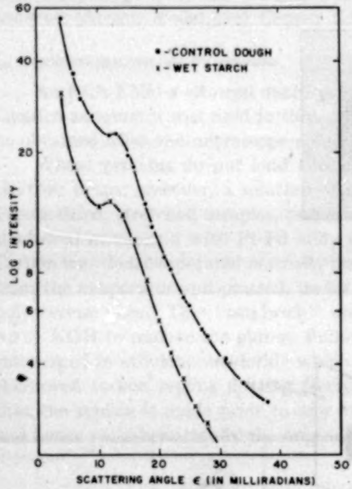


Fig. 2. Small-angle X-ray scattering from wheat flour dough and wheat starch.

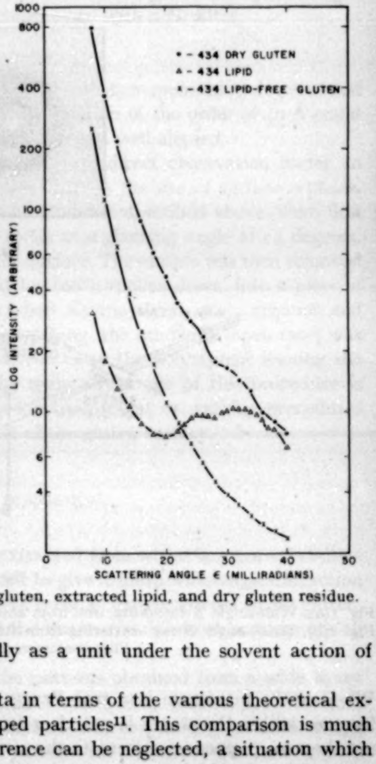


Fig. 3. Small-angle X-ray scattering from dry gluten, extracted lipid, and dry gluten residue.

It is usual to analyze small-angle data in terms of the various theoretical expressions for scattering from various shaped particles[11]. This comparison is much easier if the effects of interparticle interference can be neglected, a situation which is satisfied for dilute solutions. In order to estimate the amount of interparticle interference in the scattering from concentrated glutens, curves were obtained from dilute dispersions of gluten in acetic acid (pH = 3.2) and compared with moist gluten as obtained by the usual washing procedures. It is clear from Fig. 4 that the effects of interparticle interference are small for moist gluten, resulting mainly in a slight depression of the curve at the smaller angles. The effect can be neglected in the analysis of the tail of the curves considered in the next paragraph. (Lipid lines are visible in the 20–40 mr. range for moist gluten.)

SCHMIDT[12] has calculated the scattering from polydisperse solutions of ellipsoids of revolution. For thin platelets, the intensity is proportional to ε^{-2} at moderately large angles, while at larger angles, the dependence changes to ε^{-4}; for long rods, the scattering varies as ε^{-1} at moderately large angles, changing to ε^{-4} at larger angles. Spherical particles exhibit only the ε^{-4} dependence.

For purposes of experimental comparison, the functions ε^{-2} and ε^{-1} were integrated over the areas of the collimating and defining slits of the small-angle camera.

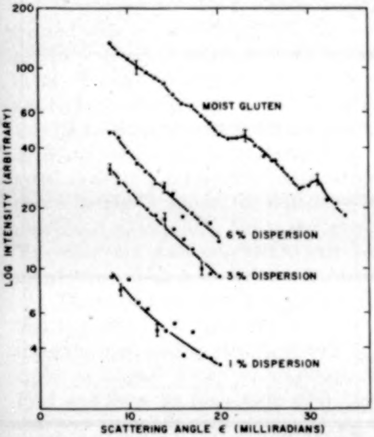


Fig. 4. Small-angle X-ray scattering from various gluten concentrations. Dispersions made in acetic acid (pH 3.2).

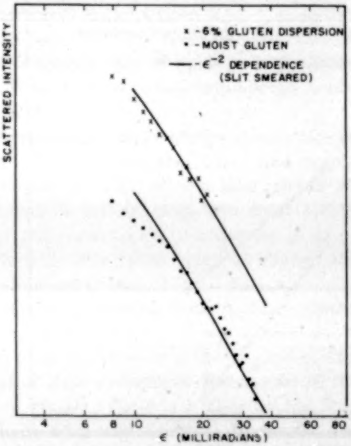


Fig. 5. Comparison of small-angle X-ray scattering from gluten with theoretical platelet, ε^{-2}, dependence.

Agreement with the ε^{-2} (platelet) dependence is shown in Fig. 5. An estimate of the platelet thickness can be determined by observing the angle at which the ε^{-2} dependence begins to change to ε^{-4}. Data for moist glutens were taken out to 100 mr. At about 50 mr. the curve began to flatten without over showing any ε^{-4} dependence. The reason for this flattening is thought to be due either to thermal diffuse scattering or to the beginnings of the leading edge of the diffuse interhelix peak whose maximum falls at ~140 mr. Nevertheless, knowing that ε^{-2} scattering persists to at least 50 mr., the statement can be made that the platelet thickness is no greater than ~70 Å.

It is well known that the small-angle scattering from particles of any shape will follow the approximate experimental law of Guinier (ref. [11], p. 26) at or near $\varepsilon = 0$. Because of the demonstrated platelet structure of gluten, one would not expect close agreement with the Guinier expression except at very small angles. Nevertheless, log I (ε^2) plots show some degree of linearity for the gluten dispersions and moist gluten (Fig. 6). The slope of the straight-line portion can be interpreted in terms of a radius of gyration whose value is 31.5 Å for the dilute dispersion. Interparticle interference effects depress the slope somewhat for moist gluten. The relation of this number to the platelet thickness is not obvious in view of the lack of information on the details of the platelet shape. Nevertheless, an approximation is to state that the thickness ~$2R_0$, or 62 Å. This is consistent with the other data yielding $t < 70$ Å.

Although dry glutens do not show, as do the moist glutens, the marked agreement with the predicted platelet scattering, several conclusions can be drawn about their structure. Firstly, the tail of the dry scattering curve falls somewhat more rapidly than ε^{-2}. This would be true for oblate ellipsoids with axial ratios larger than those found in the moist state. At first glance this appears contradictory since one would expect shrinkage rather than swelling to occur with loss of water. However, the agglomeration of several thin platelets into a single unit while drying could account for the observations. This is further supported by the behavior of the dry curves at the smaller angles. Unlike the moist glutens, there is a rapid increase in scattered intensity for $\varepsilon < 15$ mr. No good radius of gyration can be obtained from the data, but it is evident that larger values are contributing to the scattering. KRATKY AND POROD[13] have shown that scattering from lamellae in densely packed systems gives rise to a "minimum angle" scattering which increases rapidly as ε approaches zero.

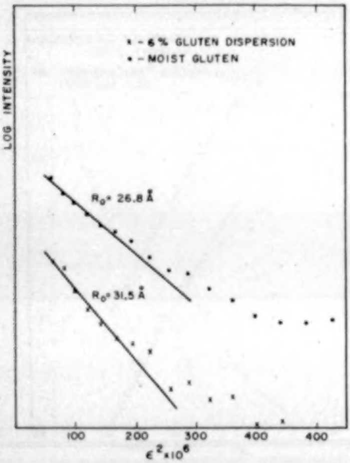


Fig. 6. Guinier plot of small-angle X-ray scattering from gluten. R_0 = radius of gyration.

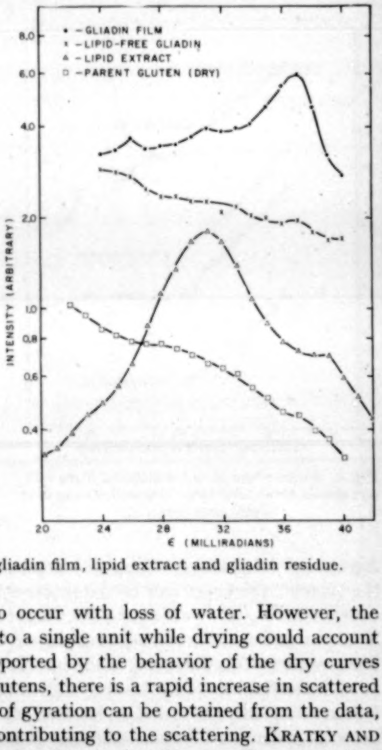


Fig. 7. Small-angle X-ray scattering from gliadin film, lipid extract and gliadin residue.

Additional evidence for the platelet structure was obtained from photographs of the X-ray scattering at small angles from stretched, freeze-dried gluten. Fig. 1(b) shows the experimental arrangement and the oriented pattern that was obtained. Clearly the platelet must be oriented flat into the plane of stretch. (Photographs taken with the X-ray beam perpendicular to the plane of stretch show a diffuse unoriented scattering with a faint oriented pattern superimposed. This latter is thought to be the result of a few platelets which are rotated to some degree with respect to the plane of stretch.) The dependence of scattered intensity on scattering angle in the plane perpendicular to the stretch has been measured using a counter and found to be the same (except for the greatly increased intensity) as that from powdered glutens. One then obtains the picture of stretched gluten existing in parallel sheets, made up of platelets which have bonded together under the action of hydration and mechanical mixing. The majority of these sheets are oriented parallel to the plane of stretch, although a few, through folding, are to be found at all angles with respect to the majority.

It was not possible to obtain an oriented small-angle pattern from glutens extracted with wet *n*-butanol. One therefore concludes that the sheet-like structure of ordinary stretched gluten owes its existence to the presence of lipid, most probably in the form of a lipo-protein complex. No significant difference was found in the X-ray scattering from the two pure wheat strains, 3237 and 3238.

(c) *Gliadins.* TRAUB *et al.*[2], reported a sharp diffraction line from gliadin film at 47 Å and attributed the interference to the lipid component which, in a film evaporated to dryness from the ethanol extract, remains with the gliadin. The uppermost curve in Fig. 7 shows the scattering obtained in this investigation from a dry, pulverized gliadin film. The main peak at 37 mr. corresponds to a spacing of 42 Å. Measurements made on gliadins extracted from six other flour types all showed the interference at 42 Å to within a few tenths of an angstrom.

The second and third curves in Fig. 8 show the scattering from lipid-free gliadin and from the lipid which was extracted with wet *n*-butanol. The main interference from the lipid falls at 31 mr., or 49.7 Å. This peak can be seen in the original gliadin curve as a small bump which disappears after lipid extraction. Extraction of the lipid also removes the strong 42 Å line from gliadin. There is a knee on the lipid curve at 42 Å, but with nothing of the intensity originally observed for gliadin. The curve from the parent (unextracted) gluten is also shown and the influence of the lipid interferences can be seen.

B. *Electron microscopy*

Electron micrographs were taken of the replicas of stretched, freeze-dried gluten surfaces. The method of preparing the replica was described in an earlier paragraph. Fig. 8(a) shows the surface of 3237 gluten at fairly low magnification (8000 ×). Notice

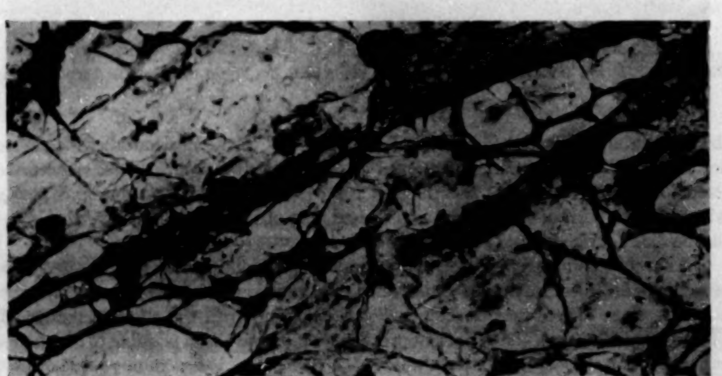


Fig. 8(a). Electron micrograph of surface of stretched, dry gluten (carbon replica 8,000 ×).

Fig. 8(b). Electron micrograph of surface of stretched, dry gluten (carbon replica 43,600 ×).

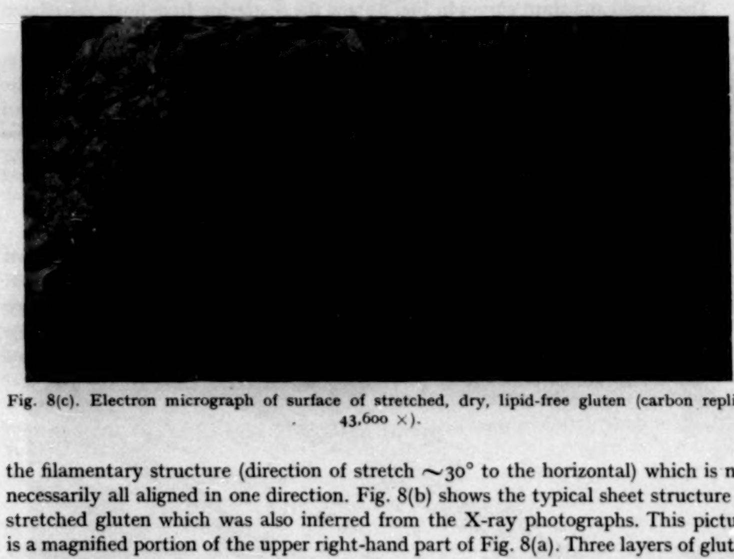


Fig. 8(c). Electron micrograph of surface of stretched, dry, lipid-free gluten (carbon replica 43,600 ×).

the filamentary structure (direction of stretch ~30° to the horizontal) which is not necessarily all aligned in one direction. Fig. 8(b) shows the typical sheet structure of stretched gluten which was also inferred from the X-ray photographs. This picture is a magnified portion of the upper right-hand part of Fig. 8(a). Three layers of gluten can be distinguished.

A careful examination of the replicas under high magnification has revealed many broad expanses of unruptured gluten sheet. In regions of high stress concentration, however, these sheets first develop holes which grow until the sheet is reduced to a network of filaments. Using present procedures it has not been possible to distinguish individual platelets in the gluten sheet. If their thickness is less than 70 Å, however, their presence would be betrayed only by undulations ~35 Å high in the surface of the gluten. This is at the limit of resolution of the EMU-2 microscope presently in use.

Lipid removal results in the gluten structure shown in Fig. 8(c). Only fragmentary "sheets" can be seen.

CONCLUSIONS

The physical structure of wheat protein as revealed by X-ray and electron microscopy has been shown to be:

1. Folded polypeptide chains in the α_1-helix configuration of PAULING AND CORY.
2. Molecular platelets of a thickness not greater than about 70 Å. Under hydration these platelets bond together into sheets which can be oriented by an applied stress parallel to one another and in the plane of stretch.
3. Dependent on the presence of lipids (wet *n*-butanol extractable) to the extent that lipid removal, while not affecting the basic platelet, destroys the ability to bond into sheets.

Gliadin films, prepared by evaporation of the ethanol extract, were shown to have a structural periodicity of 42 Å. This is apparently not a true lipid interference, but removal of the lipid destroys the periodicity. Lipids themselves all show a strong diffraction maximum at ~48 Å, with subsidiary peaks at ~42 and 62 Å.

No significant differences, other than the well-known macro-elastic properties, have been found in the microstructure of different wheat protein strains.

ACKNOWLEDGEMENTS

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STUDIES OF RATES OF ESCAPE OF PROTEINS THROUGH MEMBRANES

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SUMMARY

Rate coefficients of escape of BPA and Fraction II human γ-globulin through large pore size nitro-cellulose membranes were shown to be constant up to 1 g/100 ml.

A general scheme of statistical analysis of diffusion data for a two-component system is presented. One particular application consisted in the calculation of the original composition of a known mixture of BPA and Fraction II from the shape of the escape rate curve. The calculated concentrations agreed to within 5 % with those directly measured.

Prolonged diffusion of a concentrated solution of Fraction V human albumin during which 99.16 % had escaped, showed that all the large molecular weight impurities of Fraction V which remained "inside" had become insoluble.

INTRODUCTION

Escape* rate measurements have been shown to serve as a test for heterogeneity of small molecules[1-3]. Heterogeneity tests of this type might be of considerable value for large molecular weight substances in supplementing those used in ultracentrifugation, electrophoresis and diffusion, by virtue that, in diffusion through a membrane, small differences in size, shape and charge may be more readily demonstrable. Escape rate measurements are also likely to provide a convenient way for detecting interaction between proteins, or proteins and haptens. In the present paper the theory of the two-component problem is discussed and in part illustrated by a statistical analysis of data obtained with solutions of human plasma albumin, bovine plasma albumin, human γ-globulin and a mixture of the last two.

The use of large pore size membranes with an open cell necessitated precautions to avoid hydrostatic flow. A microtechnique for work with volumes between 0.3 and 0.5 ml is described.

MATERIALS AND METHODS

Crystalline bovine plasma albumin (BPA), a sample from the batch No. P 67403 made by Armour Co., and human albumin Fraction V and human γ-globulin Fraction II

Abbreviation: BPA, bovine plasma albumin.

* This term is used by CRAIG *et al.*[1] apparently in distinction to "dialysis" which refers to small molecules in the presence of large ones to which the membrane is impermeable.

were used. The latter two were given to us by the American Red Cross Blood Program. The buffer solution used contained 0.1 mole KCl, 0.01 mole each of sodium acetate and acetic acid and 0.0015 mole NaN_3 (= 0.1 g) per l and had a pH of 4.7. The proteins were dissolved in the buffer solution and centrifuged. Fraction II was stabilized with 1/20 of its weight of BPA.

The apparatus was the same as described earlier[3] except for the cell (Fig. 1) which had been modified for use with large pore size membranes and for much smaller volumes. The area of the exposed membrane surface was 0.754 cm^2. A Schleicher and Schuell medium Membrane Filter of 0.25 micron average and 0.5 μ maximum pore size served as membrane. Three pointed lucite pins (Fig. 1, P) were used as level indicators in the procedure of adjusting the central shaft vertical and the membrane parallel to the "outside" fluid surface. The level of the 1500 ml outside fluid was adjusted by means of a vertical glass rod, 9.1 mm in diameter, which was lowered until the surface of the fluid touched one levelling pin. This adjustment was reproducible to within 1 or 2 mm on the rod, that is, to better than 0.01 mm of the fluid level in the container, the free surface area being 164 cm^2. The water lost by evaporation was replaced at half hourly (1–2 ml) and the acetic acid at daily intervals (0.05–0.1 ml glacial acetic acid). All experiments were carried out at 25.0° with fluctuations usually of less than 0.1°. The "inside" surface of the membrane was swabbed with a small cylindrical mop of tissue paper held by a polyethylene clamp (Fig. 1, T). The sample was introduced and withdrawn from the cell by means of a capillary point polyethylene pipette. At the end of every run the weights were

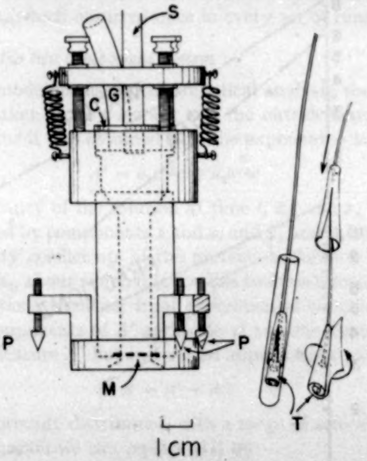

Fig. 1. Diffusion cell. M = membrane, P = levelling pins, G = polyethylene guiding tube for pipette, S = shaft, C = cork, T = tissue paper mop. The cylindrical center section has shallow concentric groves by which the membrane tightly seals the chamber. It is attached to the end pieces by three 3/16 stainless steel bolts (not drawn in the figure) the ends of which at the bottom are sealed in by a plug of lucite. Paddles for stirring the outside fluid, as shown in the earlier paper[3], are used also here, but for clarity have been omitted from the drawing. They are shaped so that on every stroke of the cell the bottom surface of the membrane is flushed.

taken of (a) the solution after transfer into a small serological test tube (50 × 6 mm), (b) the pipette before and after use and (c) the mop before and after use in order to obtain the total amount of solution at the end of the run. Preliminary tests with buffer instead of protein solution were made to determine the volume for which the hydrostatic flow was zero, that is, the volume of that amount of fluid which was recovered unchanged in weight. The same volume of protein solution was then used; this was 0.475 ml within a range of ± 0.5 μl and a precision of 0.1 or 0.2 μl. The volume changes ranged from —6.8 μl (—1.4 %) to + 8.9 μl (1.9 %); 70 % of the volume changes were within the range —4.2 to + 6.2 μl (—0.9 % and + 1.3 %). Whenever positive changes in volume occurred the values for the absorbancy (*A*) were corrected to refer to the original volume. Negative values for volume changes occurred in half the number of runs and were disregarded since they would not affect the protein concentration except secondarily by slightly raising the value for the rate coefficient. The outside concentration of protein never exceeded 0.2 % of that inside and was disregarded.

The concentrations of the proteins were determined by measuring the absorbancy at 278 mμ (half intensity band width 1 mμ) in a Beckman DU spectrophotometer with a photomultiplier attachment. The instrument was stabilized by two Sola constant voltage transformers, one before the powerpack and the other in series with the trickle charger and a variable resistor, adjusted so as to obviate the discharge of the battery. The micro-cells of 0.55 ml capacity were those manufactured by the

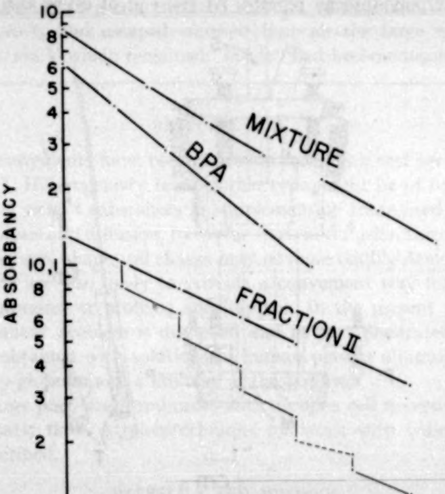

Fig. 2. Diffusion analysis of a mixture. The three upper lines were calculated to represent the amount of protein remaining inside as a function of time. The lowest line represents the actual initial and final absorbancies for each run in the Fraction II set.

Pyrocell Company, New York. As a safeguard against accidental error from light scattering of suspended particles, the solutions were routinely centrifuged for at least 30 min at 1000 × g. Three pipettes of the Pregl type, 0.0658, 0.1129 and 0.2615 ml, were employed for all dilution procedures. To the more concentrated solutions, recovered at the end of each run in the initial stages of a set of runs, 0.1129 ml outside buffer was added; 0.0658 ml of the mixture was used for analysis and the remainder for the succeeding run. The 0.0658 ml was diluted either with once or with twice 0.2615 ml outside buffer, corresponding to approximately 5 and 9 fold dilutions. To the more dilute solutions, toward the end of the set of runs, 0.0658 ml buffer was added, in order that after the absorbancy measurements sufficient solution should be available for the succeeding run. The outside buffer as reference and dilution fluid was preferred to unused buffer because it was found that the absorbancy of the buffer solution at 278 mμ, mostly contributed by NaN_3, decreased from 0.02 to about 0.012 in 5 h, to 0.008 in 9 h and then possibly somewhat further, in the presence of traces of albumin or of Fraction II. With the majority of the values for A ranging between 0.4 and 1.5 the uncertainty in the blank value did not seem to lead to serious error. Precautions were taken to minimize errors caused by vaporization of water from small samples. Further, by weighing all pipetted quantities to 0.1 mg precision, the pipetting error was reduced; for example, that of the smallest pipette was reduced to about 0.2 %. The uncertainty for the larger pipettes was correspondingly less, percentagewise, but it depended also on the values assumed for the density increments of the proteins (0.003 for a 1 % solution). Using 0.15 to 0.2 ml for each absorbancy measurement it was possible to measure all solutions in duplicate or triplicate except for the 5-fold dilutions (which occurred once in every set of runs).

Statistical inference in the two component system

In setting up the model for use in the statistical analysis, the activity coefficients of the proteins were taken to be constant and the outside concentration of protein as negligibly small. Thus it was assumed that the exponential law is obeyed[3]

$$A' = a_1 e^{-k_1 t} + a_2 e^{-k_2 t} \qquad \text{(I)}$$

where A' is the absorbancy of the solution at time t, a_1, and a_2 are the absorbancies at time zero contributed by components 1 and 2, and k_1 and k_2 are the rate coefficients and include the activity coefficients of the proteins as factors. There are four parameters, a_1, a_2, k_1, and k_2, about which it is possible to draw inferences. We can imagine several different situations resulting from experimental circumstances. We suppose in each case that measurements of A' are made at various times t.

Now we do not measure A' but instead an approximate value A where

$$A = A' + A'\varepsilon \qquad \text{(II)}$$

and ε we take to be normally distributed, with a mean of zero and constant variance σ^2. To a first approximation we can replace (II) by

$$A = A' + A\varepsilon \qquad \text{(III)}$$

Thus

$$A = a_1 e^{-k_1 t} + a_2 e^{-k_2 t} + A\varepsilon \qquad \text{(IV)}$$

is an ordinary regression model with weights

$$w = 1/A^2 \qquad \text{(V)}$$

Now (IV) can be written approximately

$$A \doteq a_1 e^{-k_{10}t} + a_2 e^{-k_{20}t} - a_1(k_1 - k_{10})te^{-k_{10}t} - a_2(k_2 - k_{20})te^{-k_{20}t} + A\varepsilon \qquad \text{(VI)}$$

where k_{10} and k_{20} are either known or trial values of k_1 and k_2. Let

$$\begin{array}{lll} x_1 = e^{-k_{10}t} & x_3 = te^{-k_{10}t} & a_3 = -a_1(k_1 - k_{10}) \\ x_2 = e^{-k_{20}t} & x_4 = te^{-k_{20}t} & a_4 = -a_2(k_2 - k_{20}) \end{array} \qquad \text{(VII)}$$

Then (VI) becomes

$$A \doteq a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4 + A\varepsilon \qquad \text{(VIII)}$$

Estimation

Maximum likelihood estimates for the four unknown parameters a_1, a_2, k_1, and k_2 are found by applying the method of least squares to the linearized model (VIII) in successive iterations. If the k's are both known $k_{10} = k_1$ and $k_{20} = k_2$ and hence $a_3 = a_4 = 0$. In this case a single cycle of iteration provides final estimates of a_1 and a_2. On the other hand if at least one k is unknown (*i.e.*, must be estimated) then preliminary estimates of a_1, a_2, a_3, and a_4 are found which also by (VII) provide improved values for k_1 and k_2. These replace k_{10} and k_{20} in the determination of the x's, using eqn. (VII), and a 2nd cycle is performed. Additional cycles are performed until stable estimates of a_1, a_2, k_1, and k_2 are obtained. These are then the maximum likelihood estimates.

In order to present a unified method of estimation for the various cases of *a priori* knowledge we define an "adjusted absorbancy" A^* as follows:

a_1, k_1, and k_2 known	$A^* = A - a_1x_1$
k_1 and k_2 known	$A^* = A$
a_1, k_1 and a_2 known	$A^* = A - a_1x_1 - a_2x_2$
a_1 and a_2 known	$A^* = A - a_1x_1 - a_2x_2$
k_1 known	$A^* = A$
a_1 known	$A^* = A - a_1x_1$
none of the parameters known	$A^* = A$
a_1 and k_1 known	$A^* = A - a_1x_1$

The method of least squares gives rise to the following "normal equations":

$$\begin{array}{l} \Sigma wx_1^2\cdot a_1 + \Sigma wx_1x_2\cdot a_2 + \Sigma wx_1x_3\cdot a_3 + \Sigma wx_1x_4\cdot a_4 = \Sigma wx_1A^* \\ \Sigma wx_1x_2\cdot a_1 + \Sigma wx_2^2\cdot a_2 + \Sigma wx_2x_3\cdot a_3 + \Sigma wx_2x_4\cdot a_4 = \Sigma wx_2A^* \\ \Sigma wx_1x_3\cdot a_1 + \Sigma wx_2x_3\cdot a_2 + \Sigma wx_3^2\cdot a_3 + \Sigma wx_3x_4\cdot a_4 = \Sigma wx_3A^* \\ \Sigma wx_1x_4\cdot a_1 + \Sigma wx_2x_4\cdot a_2 + \Sigma wx_3x_4\cdot a_3 + \Sigma wx_4^2\cdot a_4 = \Sigma wx_4A^* \end{array} \qquad \text{(IX)}$$

where the weights w are given by (V), the x's are given by (VII) and the A^*'s are the adjusted observed absorbancies. The numerical solution of the linear eqns. (IX) yields estimates of the a's which are then iterated as indicated above. Equations and terms corresponding to known parameters are omitted. For example, in the case that a_1 and k_1 are known *a priori*, the first and third equations are omitted as are the first and third terms of the remaining equations.

Confidence limits

Where possible, it is desirable to obtain a range of values within which an estimated parameter is deemed to lie. Such a range is called a confidence region and the end points of the interval are known as confidence limits. In some cases, if one states that the parameter lies within a region, an exact probability that the statement is in error can be found. Then the region is said to be an exact confidence region. Under certain restricted conditions an exact confidence region can be found that utilizes all of the information in the data concerning the parameter. This exact confidence region is termed a fiducial region and the bounds are fiducial limits. Only the fiducial limits truly represent the amount of information about the parameter inherent in the data. In still other cases no exact confidence region is easily found but instead exact limiting results for very large samples may be obtained. Limiting regions of this kind are termed asymptotic confidence regions and may be approximately correct for moderately large samples of data. Asymptotic confidence regions can be quite inaccurate for small amounts of data. The amount of data required for sufficient accuracy is not always easily determined and depends upon, among other things, the true values of the parameters.

Asymptotic results may in principle always be obtained for maximum likelihood estimators. Hence these are available for all of the estimators described in the previous section. Since it is expected that application of the techniques herein discussed will be mainly confined to relatively small samples, details of computation of asymptotic confidence regions will not be given. For these details see DEMING[4]. Exact joint confidence regions for the exponential parameters can be obtained by methods discussed by TURNER[5]. The discussion here will be limited to those cases in which the exponential parameters k_1 and k_2 are known *a priori*. In these cases fiducial regions are available and easily obtained.

The computations are carried out in stepwise fashion. First eqns. (IX) are modified after eliminating 3rd and 4th equations and terms, to

$$\begin{aligned} \Sigma w x_1^2 \cdot c_{11\cdot2} + \Sigma w x_1 x_2 \cdot c_{21\cdot2} &= 1, 0 \\ \Sigma w x_1 x_2 \cdot c_{11\cdot2} + \Sigma w x_2^2 \cdot c_{21\cdot2} &= 0, 1 \end{aligned} \tag{X}$$

The first set of numbers (1 and 0) on the right are used to obtain c_{11} and c_{21} and then the second set (0 and 1) are used to obtain c_{12} and c_{22}. Next we calculate an estimate of the error variance σ^2. This is given by

$$s^2 = \frac{1}{n-2}\left\{\Sigma w(A^*)^2 - \hat{a}_1 \Sigma w x_1 A^* - \hat{a}_2 \Sigma w x_2 A^*\right\} \tag{XI}$$

where $\hat{a}_1$ and $\hat{a}_2$ are the maximum likelihood estimates and n is the number of observations. A value of STUDENT's t with n-1 degrees of freedom is chosen at the desired confidence level (*e.g.*, 95) and confidence regions are defined by

$$\begin{aligned} \hat{a}_1 - t\sqrt{c_{11}s^2} \leqslant a_1 \leqslant \hat{a}_1 + t\sqrt{c_{11}s^2} \\ \hat{a}_2 - t\sqrt{c_{22}s^2} \leqslant a_2 \leqslant \hat{a}_2 + t\sqrt{c_{22}s^2} \end{aligned} \tag{XII}$$

Thus we state that a_1 and a_2 fall within specified intervals and these intervals have been chosen so as to coincide with a specified risk of error in making such statements.

If a_1 is known in addition to k_1 and k_2 then (X) is replaced (by proper omissions) by

$$c_{22} = 1/\Sigma w x_2^2 \tag{XIII}$$

and the divisor in (XI) becomes n-1 as does the degrees of freedom for STUDENT's t.

RESULTS

Known mixture

Table I and Fig. 1 show the results of experiments with BPA, Fraction II and a mixture of the two. The data for BPA were analyzed on the basis of eqn. (XIV)

$$A' = a_1 \mathrm{e}^{-k_1 t} \tag{XIV}$$

The curves for Fraction II and for the mixture of BPA and Fraction II show a discontinuity which was attributed to the instability of Fraction II when diluted with buffer solution. This dilution was necessary for the measurement of absorbancy. After the point of discontinuity the solutions were measured almost undiluted (1 in 1.14) and Fraction II was then assumed to be stable under the conditions of handling the samples and to obey eqn. (I). However, before the discontinuity we have postulated that, on account of the losses of Fraction II by precipitation, eqn. (I) be modified to

TABLE I

RATES OF ESCAPE OF PROTEINS THROUGH MEMBRANES

Solution 1 *10 mg/ml BPA*			*Solution 2* *10 mg/ml fraction II and 0.5 mg/ml BPA*			*Mixture* *2 ml solution 1 and 1 ml solution 2*		
Time (min)	*Absorbancy**		*Time (min)*	*Absorbancy**		*Time (min)*	*Absorbancy**	
	Obs.	*Calc.*		*Obs.*	*Calc.*		*Obs.*	*Calc.*
0.0	6.224	6.1660	0.0	12.95	13.0905	0.0	8.503	8.4304
255.0	4.219	4.1901	286.0	10.262	10.2106	422.0	5.065	5.1319
505.0	2.834	2.8690	564.5	8.075_4	8.0219	718.0	3.653	3.6643
743.5	1.999_2	1.9990	867.0	6.183	6.1762	998.0	2.708	2.6870
978.5	1.416_3	1.4002	1142.5	4.833	4.8696		*Discontinuity*	
1227.5	0.981_1	0.9602		*Discontinuity*		1284.0	2.174	2.1709
			1420.0	4.245	4.2582	1560.0	1.629_8	1.6319
			1704.0	3.346	3.3357	1843.5	1.225_5	1.2253
			0.0	—	—			
			297.0	10.176	10.1140			

* Corrected to refer to the original volume, 0.475 ml.

$$A' = a_1 \mathrm{e}^{-k_1 t} + a_2[f(\mathrm{e}^{-k_1 t}, \mathrm{e}^{-k_2 t})]\, \mathrm{e}^{-k_2 t} \tag{XV}$$

Now $f(\mathrm{e}^{-k_1 t}, \mathrm{e}^{-k_2 t})$ is unknown but can be replaced approximately by a truncated TAYLOR's series. The crudest approximation is found by taking only the constant term. Thus (XV) becomes

$$A' \text{ (before discontinuity)} = a_1 \mathrm{e}^{-k_1 t} + a_2 \beta_0 \mathrm{e}^{-k_2 t} \tag{XVI}$$

The value for a_1, which is here the absorbancy corresponding to the concentration of BPA in the "stabilized" Fraction II solution, was calculated to be equal to 0.3085. Better approximations are given of course, by including higher powers, but when only a few data-points are available a condition of relative indeterminacy arises. This means that although the model will provide a good description of the data, the determination of individual parameters will be unreliable. Eqn. (XVI) was considered appropriate* in the analysis of the experiment with the stabilized Fraction II and the mixture by assuming (a) and equal degree of instability for a 1:9 and a 1:5 dilution and (b) no precipitation by the slight dilution at the two final points in the set. The eqn. (XVI) neglects the variation in the stabilizing action of BPA, although assumption (a) implies a more stable solution in the beginning when 1:9 dilutions were used and relatively more BPA was present, than at later stages when 1:5 dilutions were used.

The magnitudes of the errors which were known to occur in the analysis were calculated on the basis of an assumed $\sigma_A = 0.001$ each in the absorbancies below 0.5 and the blank measurement, 0.002 at 0.7, 0.004 at 1.1, $\sigma_w = 0.1$ mg for each weighing in the dilution procedure and a multiplicative error caused by neglecting the effect of the volume change of the solution during the run on k, which was less than 0.5 %. From the values for the calculated errors at different stages in the experiment with BPA, shown in Table II, it seemed appropriate to postulate a multiplicative error, as was expressed in eqns. (II) and (III), rather than an additive one, in the values for the absorbancies.

TABLE II

CALCULATED MULTIPLICATIVE ERRORS AT DIFFERENT STAGES IN THE BPA SET

	Initial	*At end of run*				
		No. 1	*No. 2*	*No. 3*	*No. 4*	*No. 5*
In A, %	0.2	0.4	0.4	0.3*	0.3	0.3
In dilution factor, %	0.2	0.2	0.2	0.1	0.1	0.1

* Principally error of reading the scale.

The methods discussed in the section on estimation were extended to obtain maximum likelihood estimates for the model corresponding to eqn. (XVI). Estimates of k_1 and k_2 were determined from experiments with BPA and stabilized Fraction II alone. Since the sampling variations for these estimates are quite small, they were taken to be known in the analysis of the mixture experiment, the aim of which was the determination of the composition of the mixture. The values of the estimates obtained are given in Table IIIa. Those referring to the mixture may be compared with the values calculated from the amounts used in preparing the mixture (Table IIIb).

Heterogeneity

118 mg of Fraction V were added to 0.5 ml of buffer solution and placed in the dialysis cell over a medium membrane. After 65 h in contact with several changes of buffer solution 99.16 % of the protein (in terms of the absorbancy 278 mμ) had escaped. The residual solution which was turbid, was centrifuged and the clear

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* That no other "Accident", for example inadvertent loss of solution at the discontinuity of the mixture experiment, has introduced an error in the values after the discontinuity was shown by fitting

$$\begin{aligned} A' \text{ (before discontinuity)} &= a_1 e^{-k_1 t} + a_2 \beta_0 e^{-k_2 t} \\ A' \text{ (after discontinuity)} &= (a_1 e^{-k_1 t} + a_2 e^{-k_2 t}) r \end{aligned} \qquad \text{(XVII)}$$

to the data for varying values of r. The sum of squares was at a minimum when r was close to unity ($\hat{r} = 1.02$).

TABLE III (a)

MAXIMUM LIKELIHOOD ESTIMATES OF THE ESCAPE RATE COEFFICIENTS AND INITIAL CONCENTRATIONS

BPA		*Fraction II*		*Mixture of solutions 1 and 2*	
Parameter	*Estimate*	*Parameter*	*Estimate*	*Parameter*	*Estimate*
k_1	0.001505	k_2	0.00085	a_1	4.4897
a_1	6.166	a_2	14.209	a_2	4.5296
		β_0	0.90	β_0	0.87
		$0.3085 + a_2\beta_0 =$	13.0905	$a_1 + a_2\beta_0 =$	8.4304

(b)

ABSORBANCIES IN MIXTURE CALCULATED FROM INITIAL CONCENTRATIONS OF SOLUTIONS 1 AND 2

	From best fit curve	*From measured values*
BPA	4.2135	4.2522
Fr. II	4.7363	4.6841
Total	8.4743	8.4660
Total observed	8.503	

supernate was tested with the same membrane as used for the experiments with BPA and Fraction II. At the end of the first run the solution showed opalescence which was removed by centrifugation. After measurement of absorbancy the solution was re-run twice, each time supplemented with just sufficient initial supernate to give the required 0.5 ml for the next run. A run with a fresh solution of BPA was included in the set for comparison. The results are given in Table IV.

It was suprising to find so little difference in the values for *k* between the soluble residue and the original Fraction V since even 1 or 2 % of material of molecular weight larger than γ-globulin would have given a considerably lower *k*-value, had this material remained soluble. The conclusion that can be made from these experiments is that Fraction V contains as contaminant principally material which is easily denatured and that the residual soluble material shows the same escape rate as albumin.

TABLE IV

COMPARISON OF A DILUTE SOLUTION OF FRACTION V WITH THE RESIDUAL 0.84 % FROM A CONCENTRATED SOLUTION OF FRACTION V AFTER DIFFUSION AT pH 4.9

	Fraction V 11.3 mg/10 ml	*BPA 9.4 mg/10 ml*	*Fraction V 11.3 mg/10 ml*	*Fraction V residue*		
				Run 1	*Run 2*	*Run 3*
Day	0	4	5	6	7	8
Time (min)	300	300	300	343	360	333
k	0.00143	0.00144	0.00141	0.00118	0.00132	0.00142

DISCUSSION

Several factors are expected to have an influence on the escape rate coefficients of proteins as calculated on the basis of the observed concentrations. At high concentrations one would expect larger escape rate coefficients for the same reason one finds positive deviations from VAN 'T HOFF's law of osmotic pressure. Such a trend has not been found by the present technique at the low concentrations of protein. A change in pH away from the isoelectric point will be effective because the value for the coefficient of (free) diffusion increases through the accelerating action of the small gegenions, as also because the charge on the membrane will come into play. If protein becomes insoluble during the run, as actually occurred to a small extent with a human albumin preparation (Fraction V) after 99.16 % had escaped through a membrane, the escape rate coefficient calculated from absorbancies of centrifuged solutions must be too large.

MAURO[6] has discussed solvent transfer in diffusion and ROBBINS AND MAURO[7] have reported experiments on non-diffusional transfer through membranes using closed cells. In an open cell, as used in the present experiments, such non-diffusional transfer of solute is expected to be very much smaller, since it depends on the rate at which the hydrostatic pressure, equivalent to the osmotic pressure, is built up. Actually, with the protein concentrations used, hydrostatic flow appeared to be negligible. For a closed cell non-diffusional transfer would be expected to begin at the moment of contact between solution and solvent.

The feasibility of a mathematical analysis of the escape rate curve for a mixture of two known substances does not depend on whether the substances when by themselves obey the linear log (concentration) — time relationship, since this can be allowed for by introducing higher powers in the exponents of eqn. (XVI). It is, however, necessary to postulate that the two substances do not interact with each other, *i.e.* that they behave in the presence of each other as they behave singly. On the other hand, deviation of the experimental escape rate curve for a mixture from that calculated (known are the initial concentrations and the escape rate curves for the components) indicates interaction. Statistical tests can be constructed to quantity this deviation.

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THE PARTIAL SPECIFIC VOLUME OF MUSCLE PROTEINS*

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SUMMARY

The partial specific volume, $\bar{v}$, of the muscle proteins: rabbit myosin, rabbit tropomyosin and *Pinna nobilis* tropomyosin has been explored by the pycnometric method as a function of solvent composition and temperature. The values determined in neutral salt solutions fit the general pattern of agreement noted by McMEEKIN AND MARSHALL between observed and calculated partial specific volumes of proteins. Since this agreement depends on neglecting electrostriction in calculating $\bar{v}$, measurements were made in a non-aqueous organic solvent, xylene, and in solutions of different pH as well as in concentrated urea. The findings obtained are consistent with the view that electrostriction exists in these proteins but is presumably being masked by a compensating factor which may well be the "excluded volume" postulated by LINDERSTRØM-LANG.

Significant variations of $\bar{v}$ with temperature over the range 5–25° were observed for the 3 proteins studied, suggesting that the evaluation of molecular weights from sedimentation–diffusion data at temperatures below room temperature should also include $\bar{v}$ measurements at the same temperature.

INTRODUCTION

The calculation of molecular weights from ultracentrifuge data requires the evaluation of the term $(1 - \bar{v}\varrho)$. Since the partial specific volume, $\bar{v}$, of most proteins is generally between 0.70 and 0.75 ml/g and the solvent density, ϱ, is normally close to unity, a 1 % error in $\bar{v}$ results in one of about 3–4 % in the factor $(1 - \bar{v}\varrho)$. For this reason it is imperative that accurate values of $\bar{v}$ be obtained.

McMEEKIN AND MARSHALL[1], from the available data on amino acid analysis, have calculated partial specific volumes for several proteins, using the specific volumes of the individual amino acid residues as suggested by COHN AND EDSALL[2]. In most of the cases studied good agreement was obtained between calculated and experimentally observed values. The success of these calculations suggested that it is correct to neglect electrostriction due to the charged side chain groups in calculating the volume of a protein from that of its constituent amino acid residues. However, both EDSALL[3] and WAUGH[4] have pointed out that electrostriction must occur in proteins since most of their acidic and basic groups are readily accessible for titration and therefore presumably also for interaction with water.

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One possible explanation for this apparent absence of electrostriction in proteins might be found in the appreciable uncertainty associated with the $\bar{v}$ values for the amino acid residues (errors of the order of ± 2 %); it may partly be chance that values were chosen which fit so well the values for the proteins. Another possible explanation comes from LINDERSTRØM-LANG's work on the very large volume contractions attending the early stages of the enzymic hydrolysis of proteins[5]. This study suggests that the framework of the native protein structure is held in a configuration which contains a certain amount of empty space in the interior of the molecule and which is inaccessible to solvent. This so-called "excluded volume" may be compensating for the effects of electrostriction. CHARLWOOD[6], using the magnetic float method, confirmed the presence of the excluded volume in edestin, serum albumin and ovalbumin and showed that it may alter in size under different experimental conditions.

The present study reports the effect of solvent composition and temperature on the $\bar{v}$ values of the muscle proteins: rabbit myosin, rabbit tropomyosin and an invertebrate tropomyosin isolated from the mollusc, *Pinna nobilis*. These proteins were chosen because in each case there is a high ratio of charge to mass (~300 charged residues/10^5 g protein) so that the effects of electrostriction should be very pronounced. In particular it was thought that these proteins might well prove to be the exception to the MCMEEKIN-MARSHALL rule of calculating $\bar{v}$ values without correcting for electrostriction.

The temperature variable was explored to test the reliability of the assumption, usually made, that $\bar{v}$ varies little with temperature within the range 0–25°. If there was any significant variation this could be a serious source of error in molecular weight determinations from ultracentrifugal data. This is particularly true with myosin whose sedimentation and diffusion properties must be determined below 18° in view of its tendency to aggregate at room temperature[7].

EXPERIMENTAL

Myosin was prepared from rabbit muscle by the procedure of MOMMAERTS AND PARRISH[8] which incorporates the essential features of the methods of SZENT-GYÖRGYI[9] and of PORTZEHL, SCHRAMM AND WEBER[10]. It was crystallized by dilution of freshly prepared solutions with water and the precipitates were dialyzed against water at 0° until the protein swelled to a gel. This dialysis was continued at 4° for 1 week. The salt-free gel was dried over P_2O_5, first in the cold, then *in vacuo* at room temperature, and finally after being powdered at 110° *in vacuo* over P_2O_5 until its weight was constant. Heating was thus avoided until essentially all water had been removed. When such preparations were dissolved in 0.5 *M* KCl buffered with phosphate at pH 7 to a total ionic strength of 0.6 they were found to approach complete purity as judged by ultracentrifugal analysis.

Tropomyosin was prepared from rabbit muscle by BAILEY's method[1] and stored at 0° as an ethanol–ether dried powder. Material prepared this way was shown to be electrophoretically homogeneous at pH 7.

Some measurements are also reported on invertebrate tropomyosin samples kindly supplied by Dr. K. BAILEY who prepared them from the whole adductors of *Pinna nobilis* by the ethanol method[12]. These samples gave good electrophoretic and analytical criteria of purity[13].

Stock protein solutions of a definite concentration were made up by direct weight of the dry protein in whatever solvent system was being explored. These were always checked interferometrically by measuring the refractive index difference between solution and solvent. This was in fact the only satisfactory method for determining protein concentrations in concentrated urea solutions. Before this could be done, it was necessary to determine the specific refractive index increments of the proteins in the principal solvent systems used. For this purpose a Phoenix precision differential refractometer was used and this was calibrated by means of data for KCl solutions at 5461 Å[14,15]. Refractometer measurements at 5780 Å and parallel dry weight experiments led to the figures shown in Table I.

TABLE I

SPECIFIC REFRACTIVE INCREMENTS AT 5780 Å AND 25°

Protein	*Solvent system*	*Specific refractive increment* (g^{-1} cm^3)
Rabbit tropomyosin	KCl–phosphate buffer (pH 7, I = 0.6)	0.188 ± 0.001
	8 M urea in KCl–phosphate buffer (pH 7, I = 0.6)	0.155 ± 0.001
Pinna tropomyosin	KCl–phosphate buffer (pH 7, I = 0.6)	0.188 ± 0.001
	8 M urea in KCl–phosphate buffer (pH 7, I = 0.6)	0.156 ± 0.001
Rabbit myosin	KCl–phosphate buffer (pH 7, I = 0.6)	0.189 ± 0.001

Solutions of the muscle proteins were measured in capped pycnometers of about 10 ml capacity which were calibrated with air-free distilled water. Weighings were made on a Bunge balance and a Sartorius semi-micro balance (sensitivity 0.01 mg) and great care was taken to duplicate weighing conditions. Partial specific volumes were calculated from the equation[16]:

$$1 - \bar{v}\varrho = \frac{1 - w_2}{m} \cdot \frac{\mathrm{d}m}{\mathrm{d}w_2} \qquad (1)$$

where m is the mass of solution, w_2 is the weight fraction of the protein (0.01 × the concentration by weight) and ϱ is the density of the solution.

In the case of the density measurements in xylene, protein, which had been dried at 110° *in vacuo*, was weighed by difference into the pycnometer and immediately covered with the organic solvent which had been previously dried. In all, the protein was exposed to the atmosphere no longer than 10 to 15 sec. The partly filled pycnometer was then evacuated to remove dissolved and occluded gases and to facilitate complete wetting of the protein.

Temperature regulation was achieved through the use of an Aminco mercury-in-glass thermoregulator model No. 4204. Electrical contact was made as the mercury column reached the upper platinum contact wire. The water bath contained a centrifugal type pump which circulated the bath fluid past a 700 W low-lag immersion heater. A Beckman thermometer was used as a quick check on temperature fluctuations and these were steady to ± 0.01° during measurements.

Temperatures below 20° were achieved using a cooling coil which operated from a 0.25-h.p. hermetically sealed condensing unit. In order to maintain ± 0.01° accuracy at the lower temperatures, the thermoswitch operating the condensing unit was set below the desired temperature and the immersion heater, controlled by a Bronwill contact thermometer and electronic relay, was used to obtain fine temperature adjustments.

RESULTS AND DISCUSSION

The partial specific volumes of the muscle proteins in the various solvents and at the different temperatures were determined by plotting the mass, m, of a given volume of protein solution against the weight fraction, w_2, of protein. Fig. 1 shows a typical plot of the data obtained for rabbit tropomyosin in KCl–phosphate buffer (pH 7, I = 0.6) at 25 ± 0.01°. The slope of the line, dm/dw_2, was drawn according to the method of least squares and when used in equation (I) gave a value of 0.736 ± 0.002. A complete list of the v values obtained for the muscle proteins as a function of solvent composition is given in Table II. These values are estimated to be accurate to ± 0.002 and were independent of protein concentration in all the cases studied.

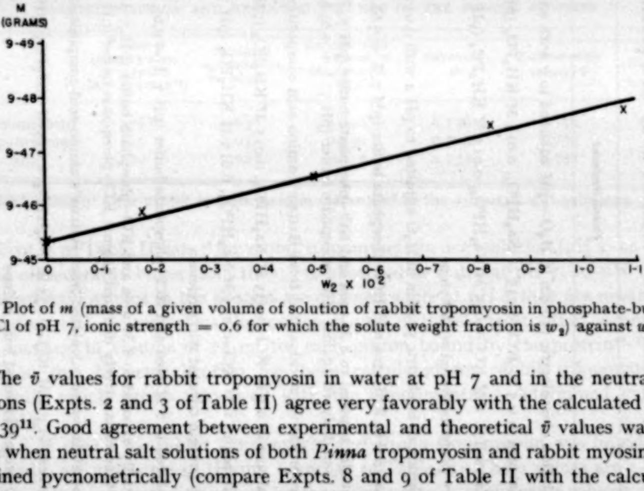

Fig. 1. Plot of m (mass of a given volume of solution of rabbit tropomyosin in phosphate-buffered KCl of pH 7, ionic strength = 0.6 for which the solute weight fraction is w_2) against w_2.

The $\bar{v}$ values for rabbit tropomyosin in water at pH 7 and in the neutral salt solutions (Expts. 2 and 3 of Table II) agree very favorably with the calculated value of 0.739[11]. Good agreement between experimental and theoretical $\bar{v}$ values was also found when neutral salt solutions of both *Pinna* tropomyosin and rabbit myosin were examined pycnometrically (compare Expts. 8 and 9 of Table II with the calculated value[17] of 0.729 and Expt. 13 with the value[11] of 0.735). This suggests that with these proteins no serious error is incurred in evaluating $\bar{v}$ from the specific volumes of their constituent amino acid residues without taking into account the effects of electrostriction.

However, that electrostriction is occurring in aqueous solutions of the muscle proteins is shown by the experiments carried out in xylene. Comparison between the values of $\bar{v}$ in water or neutral salt solution and in xylene would indicate a volume constriction of solvent water of 0.040 ml, 0.030 ml and 0.028 ml for rabbit tropomyosin, *Pinna* tropomyosin and rabbit myosin respectively. One can also calculate the degree of electrostriction from the number of ionizing groups in the molecule[18], assuming a volume decrease of 20 ml per charge pair per mole[3]. The results of this calculation are summarized in column 3 of Table III from which it is seen that

TABLE II

PARTIAL SPECIFIC VOLUME OF MUSCLE PROTEINS MEASURED AT 25° AS A FUNCTION OF SOLVENT COMPOSITION

Protein	*Experiment number*	*Protein treatment*	$\bar{v}$
Rabbit tropomyosin	1	Solution of isoelectric protein in H_2O—pH adjusted to 7 with 0.01 *N* NaOH	0.732 ± 0.002
	2	Dialyzed in 0.5 *M* KCl–0.035 *M* Na_2HPO_4, 0.015 *M* KH_2PO_4 (pH 7, I = 0.6)	0.736
	3	Dialyzed in 1 *M* KCl–0.035 *M* Na_2HPO_4, 0.015 *M* KH_2PO_4 (pH 7, I = 1.1)	0.739
	4	Dry protein covered with xylene	0.772
	5	Solution of isoelectric protein in H_2O adjusted to pH 2 with 0.01 *N* HCl	0.752
	6	Dialyzed in 8 *M* urea and KCl–phosphate buffer (pH 7, I = 0.6)	0.728
	7	Initially exposed to 8 *M* urea and KCl–phosphate buffer (pH 7, I = 0.6) then dialyzed against KCl–phosphate buffer (pH 7, I = 0.6) alone until free of urea	0.734
		Theoretical value calculated from BAILEY's amino acid composition figures[11]	0.739
Pinna tropomyosin	8	Dialyzed in 0.5 *M* KCl–0.035 *M* Na_2HPO_4, 0.015 *M* KH_2PO_4 (pH 7, I = 0.6)	0.730
	9	Dialyzed in 1 *M* KCl–0.035 *M* Na_2HPO_4, 0.015 *M* KH_2PO_4 (pH 7, I = 1.1)	0.733
	10	Dry protein covered with xylene	0.762
	11	Dialyzed in 8 *M* urea and KCl–phosphate buffer (pH 7, I = 0.6)	0.727
	12	Initially exposed to 8 *M* urea and KCl–phosphate buffer (pH 7, I = 0.6) then exhaustively dialyzed against KCl–phosphate buffer (pH 7, I = 0.6) alone until free of urea	0.731
		Theoretical value calculated from BAILEY's amino acid composition figures[17]	0.729
Rabbit myosin	13	Dialyzed in 0.5 *M* KCl–0.035 *M* Na_2HPO_4, 0.015 *M* KH_2PO_4 (pH 7, I = 0.6)	0.732
	14	Dry protein covered with xylene	0.760
		Theoretical value calculated from BAILEY's amino acid composition figures[11]	0.735

excellent agreement is obtained between the calculated and experimentally observed electrostriction.

The fact that this electrostriction is not manifesting itself in the observed $\bar{v}$ values may be due to its being masked by a compensating factor such as the "excluded volume" suggested by LINDERSTRØM-LANG's work[5]. Table III also includes estimates of the excluded volume obtained by taking the observed $\bar{v}$ and subtracting the calculated $\bar{v}$ less the calculated electrostriction. The excluded volumes so deduced correspond to 4–5 % of the observed partial molal volumes for the 3 muscle proteins; furthermore, they agree very favorably with both the calculated and experimentally observed electrostrictions. These values are consistent with recent optical rotatory dispersion studies on the muscle proteins which suggest that the greater part of their molecules is folded in the form of an α-helix[19, 20]. Such extensive folding would no doubt render a small portion of the total volume of the molecule inaccessible to solvent[4].

TABLE III

ELECTROSTRICTION AND EXCLUDED VOLUMES OF THE MUSCLE PROTEINS

Protein	*Number of charged groups/10^5 g protein (after* TRISTRAM[18]*)*	*Calculated electrostriction (ml/g)*	*Experimentally observed electrostriction (ml/g)*	*Calculated $\bar{v}$*	*Experimentally observed $\bar{v}$*	*Calculated excluded volume (ml/g)*
Rabbit tropomyosin	376	0.038	0.040	0.739	0.736*	0.035
Pinna tropomyosin	315	0.032	0.030	0.729	0.732	0.035
Rabbit myosin	270	0.027	0.028	0.735	0.732	0.024

* Mean value of $\bar{v}$ for rabbit tropomyosin in water and in the 2 neutral salt solutions.

Expt. 5 of Table II lists $\bar{v}$ for rabbit tropomyosin in acid solution (pH 2) as 0.752, a value considerably larger than the 0.732 observed in water at pH 7. At pH 7 most of the carboxyl groups in the protein are charged while at pH 2 they are neutralized by reaction with protons. This elimination of part of the electrostriction corresponds to an increase in volume of 11 ml for each proton bound by the protein[21, 22]. The expansion due to neutralization has been calculated for rabbit tropomyosin as 0.025 ml/g which agrees very well with the experimentally determined value of 0.020 ml/g.

In 8 *M* urea $\bar{v}$ for rabbit tropomyosin and *Pinna* tropomyosin was found to be 0.728 and 0.726 respectively (Expts. 6 and 11 of Table II). These values are 1–1.5 % lower than the corresponding values for these two proteins in the same solvent system in the absence of urea. This decrease in volume is consistent with the view that in urea some unfolding of the protein molecule is taking place thereby eliminating a portion of the excluded volume and facilitating penetration by the solvent. The total volume contraction observed is 440 ml/mole with rabbit tropomyosin and 675 ml/mole with *Pinna* tropomyosin. Volume contractions of the same order of magnitude have been observed to accompany the destruction of the native configuration in other proteins[6, 21, 23, 24].

It seemed of some importance to determine whether the observed changes in $\bar{v}$ in 8 *M* urea were reversible. Accordingly, solutions of *Pinna* tropomyosin and rabbit tropomyosin were dialyzed exhaustively against KCl–phosphate buffer (pH 7, I = 0.6) until free of urea and then studied pycnometrically. The $\bar{v}$ values of rabbit and *Pinna* tropomyosin were 0.734 and 0.731 respectively, in good agreement with 0.736 and 0.730 for the same proteins taken up in KCl–phosphate buffer directly. This suggests the essential reversibility of the urea treatment of these proteins, insofar as the criterion of pycnometry is concerned. A similar conclusion based on optical rotation and viscosity studies has been reached with *Pinna* tropomyosin[20].

The variation of $\bar{v}$ with temperature is summarized in Table IV.

TABLE IV

THE PARTIAL SPECIFIC VOLUME OF MUSCLE PROTEINS AS A FUNCTION OF TEMPERATURE

All measurements carried out in solvent medium of 0.5 *M* KCl, 0.035 *M* Na_2HPO_4, 0.015 *M* KH_2PO_4 (pH 7, I = 0.6).

Protein	*Temperature of measurement (C)*	$\bar{v}$
Rabbit tropomyosin	5	0.724
	15	0.730
	25	0.736
Rabbit myosin	5	0.720
	15	0.725
	25	0.732
Pinna tropomyosin	5	0.719
	15	0.725
	25	0.730

The data show that with the 3 proteins considered there is a variation of 1.5–1.7 % between $\bar{v}$ at 5° and $\bar{v}$ at 25°. Since errors in $\bar{v}$ are essentially multiplied by a factor of 3 in the final calculation of molecular weights, the above variations would result in errors of the order of 5 % in the molecular weight values deduced from the Svedberg equation. The results of these measurements therefore cast some doubt on the validity of the assumption that $\bar{v}$ varies little with temperature and suggest that the evaluation of molecular weights from sedimentation and diffusion data at temperatures below room temperature should also include $\bar{v}$ measurements at the same temperature.

ACKNOWLEDGEMENTS

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ON THE CONVERSION OF FRUCTOSE TO GLUCOSE BY GUINEA PIG INTESTINE

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SUMMARY

Inverted intestinal sacs of the guinea pig, but not of the rat, convert fructose to glucose during its passage through the intestinal wall. An exchange of ^{14}C-activity between C-1 and C-6 of the glucose formed from $[1\text{-}^{14}C]$ fructose under these conditions has been observed. Fructokinase and glucose-6-phosphatase have been found to be present in the intestinal mucosa of the guinea pig and it has been confirmed that the latter enzyme is absent in the same tissue of the rat. These observations lead to the conclusion that the conversion of fructose to glucose by the guinea pig intestinal mucosa occurs by the same mechanism as that operating in the liver, involving fructose-1-phosphate and triose phosphates as intermediates. It is suggested that the inability of rat intestine to convert fructose to glucose is due to the absence of glucose-6-phosphatase.

INTRODUCTION

It has been recognized for some time that a conversion of fructose to glucose occurs during intestinal absorption of fructose[1-4]. The mechanism involved in this conversion has not, however, been clarified and several pathways may be considered. The first one, which occurs in liver[5], involves fructose-1-phosphate, triose phosphates, fructose-diphosphate and hexose-6-phosphates as shown below:

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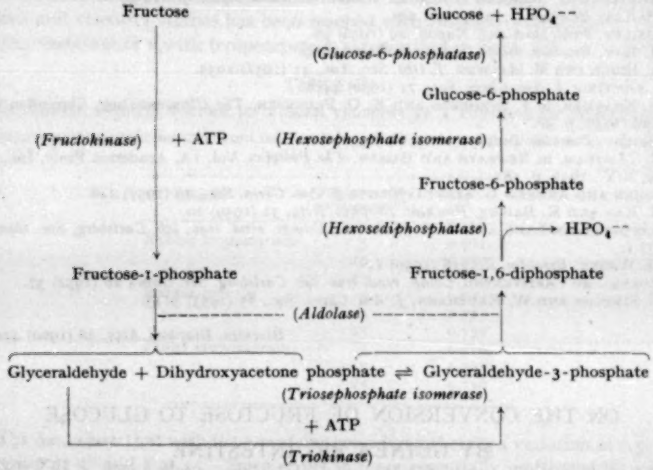
Fructose
Glucose + HPO$_4^{--}$
(*Glucose-6-phosphatase*)
Glucose-6-phosphate
(*Fructokinase*)
+ ATP
(*Hexosephosphate isomerase*)
Fructose-6-phosphate
(*Hexosediphosphatase*)
HPO$_4^{--}$
Fructose-1-phosphate
Fructose-1,6-diphosphate
(*Aldolase*)
Glyceraldehyde + Dihydroxyacetone phosphate ⇌ Glyceraldehyde-3-phosphate
(*Triosephosphate isomerase*)
+ ATP
(*Triokinase*)

A second possibility, which has some similarity with the first one, is a direct phosphorylation of fructose to fructose-6-phosphate by hexokinase, thus by-passing the triose phosphates. A third possibility is that an oxidation–reduction is involved in the conversion, with an intermediate such as glucosone or sorbitol. The latter compound is believed to be an intermediate in the reverse transformation in seminal vesicles[6]. Finally, the fourth pathway which may be considered, is a direct isomerization of fructose to glucose similar to the reaction catalysed by the xylose isomerase of *Pseudomonas hydrophila*[7].

The first pathway can be characterized by a randomization of ^{14}C-activity in glucose formed from specifically labeled fructose, due to the intermediate formation of triose phosphates[8]. It requires the presence of fructokinase and of glucose-6-phosphatase. The latter enzyme, however, could not be detected in the intestine of the rat[9]. The other pathways do not involve splitting of the hexose molecule, but the second one also requires glucose-6-phosphatase to allow the selective liberation of glucose from a mixture of hexose phosphates. If route 3 were operative, one might expect sorbitol or glucosone to be converted to glucose by the mucosa. Route 4 is a one-step reaction that could possibly be detected in a tissue extract.

This paper describes experiments designed to distinguish between the four pathways given above. The conversion of fructose to glucose by the intestine was conveniently studied by using the *in vitro* technique of WILSON AND WISEMAN[10]. As published in a preliminary communication[11], the results obtained by this technique favour the first pathway. This conclusion was supported by enzymic studies on cell-free extracts of the mucosa. In a recent paper, SALOMON AND JOHNSON[12] arrived independently at a similar conclusion.

MATERIALS AND METHODS

Sugars

Glucosone was kindly supplied by Dr. S. BAYNE of the University of St. Andrews. Uniformly labeled fructose, [1-^{14}C]glucose, [6-^{14}C]glucose and [1-^{14}C]sorbitol were purchased from The Radiochemical Centre, Amersham. [1-^{14}C]Fructose was prepared from [1-^{14}C]sorbitol by incubation with sheep seminal vesicle slices under O_2[13]. The fructose formed was purified from the incubation mixture by paper chromatography with water-saturated phenol. To prepare [6-^{14}C]fructose, [6-^{14}C]glucose was converted to a mixture of glucose-6-phosphate and fructose-6-phosphate by commercial hexokinase and phosphohexoisomerase (Sigma) and the hexoses were liberated by the action of alkaline phosphatase. Microsomes from sheep kidney were used as a source of this enzyme. The free sugars were then separated by paper chromatography with water-saturated phenol and butanol–acetic acid–water as solvents[14].

Analytical methods

Inorganic phosphate was determined by the method of FISKE AND SUBBAROW[15], lactic acid by the method of BARKER AND SUMMERSON[16], glucose by the use of glucose oxidase[17] or by its reducing power[18] and fructose by the resorcinol method[19].

The distribution of ^{14}C in the glucose samples was determined by *Leuconostoc mesenteroides* fermentation followed by chemical degradation of the products[8].

Preparation of biological material

Inverted intestinal sacs were prepared from the small intestines of guinea pigs and rats by the method described by WILSON AND WISEMAN[10]. The sacs, approximately 10 cm long, were filled with 1.5 ml of the bicarbonate buffer of KREBS AND HENSELEIT[20] and placed in the same buffer containing the appropriate sugar. After incubation in a Dubnoff shaker at 37° in an atmosphere of 95 % O_2 and 5 % CO_2, the sacs were removed and washed with water. Chemical analyses were carried out on the sac contents after deproteinization by the method of SOMOGYI[21]. When the isolation of glucose was desired, the sac contents were passed directly through a column of Amberlite MB-3 and reduced to a small volume in a vacuum desiccator. The glucose was then isolated by paper chromatography with butanol–acetic acid–water[14].

The particulate fraction from intestinal mucosa was prepared as follows: The mucosa was scraped off with a blunt spatula and 1.5 g of the scraped tissue was suspended in 9.0 ml of 0.25 *M* sucrose. After homogenizing in a Potter–Elvehjem apparatus[22], the preparation was centrifuged at 25,000 rev./min for 6 min in a Spinco Model L centrifuge, using a No. 40 head. The cloudy supernatant solution was decanted and recentrifuged for 30 min at 38,000 rev./min. The supernatant solution was discarded and the pellet suspended in 3 ml of H_2O.

RESULTS

I. Fructose to glucose conversion by the isolated intestinal sac

Species difference. Although *in vitro* experiments by DARLINGTON AND QUASTEL[2] and by WILSON AND VINCENT[3] have shown that isolated guinea pig or hamster intestines readily convert fructose to glucose during its absorption, the results presented by KIYASU AND CHAIKOFF[4] indicate that such a conversion is of much less importance in the rat. The data presented in Table I show a qualitative difference in the fate of fructose absorbed by intestinal sacs of guinea pig and rat. While glucose is the major product appearing inside the guinea pig sac, little, if any, is formed by the rat.

Experiments with radioactive sugars. Guinea pig sacs were incubated with [1-^{14}C]fructose and [1-^{14}C]glucose and the radioactive glucose samples appearing inside the sacs were isolated. The samples were then degraded in order to determine their distribution of ^{14}C-activity. The results are given in Table II.

TABLE I

FRUCTOSE, GLUCOSE AND LACTIC ACID RECOVERED FROM INTESTINAL SACS AFTER THE ABSORPTION OF FRUCTOSE

The intestinal sacs were incubated for 2 h in 3 ml of buffer containing 9 mg fructose.

Intestinal sac	*Fructose absorbed (µg)*	*Product appearing in sacs (µg)*		
		Fructose	*Glucose*	*Lactic acid*
Guinea pig	4,860	92	830	251
Rat	3,260	354	< 6	492

TABLE II

PERCENTAGE DISTRIBUTION OF ^{14}C IN GLUCOSE RECOVERED FROM GUINEA PIG INTESTINAL SACS

Intestinal sacs were incubated for 2 h in 2 ml of buffer containing 2 mg [1-^{14}C]fructose or [1-^{14}C]glucose. The radioactive glucose appearing inside the sacs was isolated and degraded as described under METHODS. The values in the table are expressed as percentages of the total activity in the glucose samples as determined by persulfate oxidation[31].

Sugar supplied	*Carbon atom*					*Recovery of activity*
	1	*2 + 3*	*4*	*5*	*6*	
[1-^{14}C]Fructose	55	2	0	0	51	108
[1-^{14}C]Glucose	97	2	0	0	1	100

It can be seen that the ^{14}C-activity in the glucose formed from [1-^{14}C]fructose is almost equally distributed between C-1 and C-6. Since no randomization occurred in the glucose during its transport across the intestinal wall, it is reasonable to assume that the observed randomization when [1-^{14}C]fructose was used occurred prior to the formation of glucose. TAYLOR AND LANGDON[23] have shown that no redistribution of ^{14}C-activity occurred in liver glycogen formed by the rat from [1-^{14}C]glucose administered by stomach tube.

These data support the first pathway, which involves the formation of glyceraldehyde and triose phosphate. The nearly equal degree of labeling found in positions 1 and 6 might be taken as an indication that the newly formed glucose arises only from the top half of fructose and that the glyceraldehyde formed is not utilized for glucose formation. In order to check this possibility, intestinal sacs were incubated with uniformly labeled fructose or fructose specifically labeled in carbon 1 or 6. Glucose appearing inside the sac was isolated and its specific activity was determined

TABLE III

SPECIFIC ACTIVITY OF GLUCOSE FORMED FROM [1-^{14}C]FRUCTOSE, [6-^{14}C]FRUCTOSE, OR UNIFORMLY LABELED FRUCTOSE BY GUINEA PIG INTESTINAL SACS

Intestinal sacs were incubated for 2 h in 2 ml of buffer containing 2 mg of the radioactive fructose samples. The glucose appearing inside the sacs was isolated as described under METHODS. The values in the table are expressed as percentage of the specific activity of the fructose supplied and represent the mean of different experiments. The standard error of mean is also given, and the figures in brackets represent the number of experiments.

Sugar supplied	*Specific activity of glucose formed*
[1-^{14}C]Fructose	122 ± 16.6 [5]
[6-^{14}C]Fructose	81.5 ± 10 [2]
[U-^{14}C]Fructose	73 ± 3.5 [5]

and compared with that of the fructose substrates. The results are shown in Table III. Although the large experimental errors preclude a quantitative evaluation of the data, it appears that C-6 of the fructose molecule is readily utilized for glucose formation. The significantly higher activities in the glucose obtained from [1-^{14}C]fructose (in some cases higher than the original fructose) would seem to indicate that not all of the glyceraldehyde arising from carbon atoms 4, 5 and 6 of fructose is metabolized by phosphorylation to triose phosphate. The recent observation by LAMPRECHT AND HEINZ[24] that D-glyceraldehyde can be metabolized in the liver via glyceric acid is of interest in this regard.

Experiments with glucosone and sorbitol. No glucose was formed inside or outside the sacs when sorbitol or glucosone were used as substrates in the incubation mixture. However, when glucosone was added simultaneously with glucose, a larger amount of glucose was recovered inside and outside the sac. This phenomenon was interpreted as being due to the inhibition of hexokinase by glucosone[25], with a resulting decrease in glucose utilization.

II. Experiments with cell-free extracts

The conversion of glucose to fructose by the first pathway requires the action of fructokinase and glucose-6-phosphatase. These two enzymes, in contrast to the other enzymes participating in this pathway, have a limited distribution in tissues. Experiments were carried out in order to determine whether they are present in the intestinal mucosa of guinea pigs.

Fructokinase. Fructose-1-phosphate, which is the product of fructokinase, has been isolated by KJERULF-JENSEN[26] from the intestinal mucosa of rats and rabbits, where it accumulates during the course of fructose absorption. A fructokinase is known to be present in the intestinal mucosa of the rat but could not be demonstrated in the cow[27]. No published data were found for the guinea pig.

The phosphorylation of fructose by ATP, catalysed by an extract of guinea pig intestinal mucosa, was tested in the presence and absence of glucose. As shown in Table IV the phosphorylation of fructose was not inhibited by glucose. This is presumptive evidence for the presence of a fructokinase, since the phosphorylation of fructose by hexokinase is almost completely inhibited by an equimolar concentration of glucose[28].

The fructose phosphate formed by the mucosal preparation was isolated as a barium precipitate in ethanol and tested for its lability in acid. The results of this experiment are given in Table V. It was found that 96 % of the esterified fructose was liberated as free sugar in 20 min at 100° in *N* HCl. This lability is characteristic of fructose-1-phosphate as contrasted with fructose-6-phosphate, which is only hydrolysed approximately 18 % under these conditions[19].

TABLE IV

EFFECT OF GLUCOSE ON THE PHOSPHORYLATION OF FRUCTOSE BY A CELL-FREE EXTRACT OF GUINEA PIG INTESTINAL MUCOSA

The reaction mixture contained 2.2 µmoles fructose, 8 µmoles ATP, 8 µmoles $MgCl_2$, 900 µmoles KCl, 20 µmoles cacodylate buffer, pH 7, 100 µmoles KF and 1 ml of a 20 % aqueous homogenate of intestinal mucosa in a total volume of 2 ml. After 40 min incubation at 37°, the mixtures were deproteinized by the method of SOMOGYI[21] and fructose was measured in the protein-free filtrate. As fructose was not metabolized in the absence of ATP, fructose disappearance was taken as a measure of fructose phosphorylation.

Glucose added (µmoles)	*Fructose phosphorylated (µmoles)*
—	1.80
0.44	1.76
2.2	1.70
4.4	1.63
11	1.82
22	1.81

TABLE V

RATE OF HYDROLYSIS OF THE FRUCTOSE PHOSPHATE FORMED BY A CELL-FREE EXTRACT OF GUINEA PIG INTESTINAL MUCOSA

Fructose phosphate was enzymically prepared by the experimental conditions given in Table IV. Deproteinization was carried out by the addition of perchloric acid. After filtration, the acid extract was neutralized with KOH and refiltered. The phosphate esters were then precipitated by the addition of $BaCl_2$ and 2 volumes ethanol. The barium precipitate was washed 3 times with 60 % ethanol and then dissolved in 1 *N* HCl. This solution was heated at 100° and aliquots were withdrawn at appropriate times for free fructose analyses after adsorption of the remaining phosphate esters by $Ba(OH)_2$–$ZnSO_4$[22]. Total fructose was determined in an aliquot not treated with the barium–zinc reagent.

Time of hydrolysis (min)	*Total fructose liberated (%)*
5	59
10	81
20	96
40	95

Glucose-6-phosphatase. Glucose-6-phosphatase, a microsome-bound enzyme[29], is known to be present in liver and kidney. It could not be found, however, in the intestinal mucosa of the rat[9]. In confirmation of this observation, the results presented in Fig. 1 show that microsomes from rat intestinal mucosa do not hydrolyse glucose-6-phosphate more rapidly than glucose-1-phosphate. In contrast, guinea pig microsomes hydrolyse glucose-6-phosphate faster than glucose-1-phosphate. The greatest difference in rate of hydrolysis was at pH 6.0, which is the pH optimum of liver glucose-6-phosphatase[30].

In view of the large difference between the glucose-6-phosphatase activity of microsomes obtained from rat and guinea pig intestinal mucosa, it was of interest to examine microsomes obtained from the mucosa of other animals for this enzymic activity. The rabbit was found to resemble the guinea pig in that a peak of glucose-6-phosphatase activity at pH 6.0 was noted. The hamster was found to have no clear-cut peak at this pH but the hydrolysis of glucose-6-phosphate proceeded 2 to 3 times more rapidly than that of glucose-1-phosphate in the range of pH 5–7, while in the pH range of 8–10, glucose-1-phosphate was hydrolysed more rapidly than glucose-6-phosphate. Microsomes from human intestinal mucosa were found to contain acid and alkaline phosphatase activities but no evidence for a glucose-6-phosphatase peak at pH 6.0 was obtained.

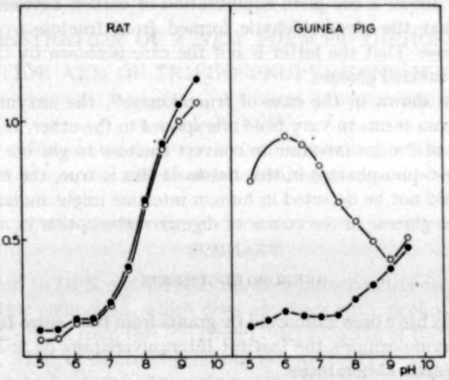

Fig. 1. Phosphatase activity of microsomes obtained from rat and guinea pig intestinal mucosa. 10 µmoles of glucose-6-phosphate or glucose-1-phosphate, 100 µmoles of buffer and a suspension of microsomes were incubated for 1 h at 37° in a total volume of 0.55 ml. Acetate was used as a buffer for pH 5.0, an equimolar mixture of tris(hydroxymethyl)aminomethane and cacodylate for pH 5.5–9.0 and glycine for pH 9.5. The microsomes in each incubation mixture were obtained from 5 mg of mucosa in the case of the rat and 25 mg of mucosa in the case of guinea pig. ○, glucose-6-phosphate; ●, glucose-1-phosphate.

Other experiments. Negative results were obtained in attempts to show either a reduction of glucosone to glucose in the presence of TPNH or an oxidation of sorbitol in the presence of methylene blue by cell-free preparations of guinea pig intestinal mucosa. It was also not possible to demonstrate a direct isomerisation of fructose to glucose.

DISCUSSION

The data presented in this paper allow a choice between several possible routes for the conversion of fructose to glucose by guinea pig intestine. The randomization of ^{14}C-activity between positions 1 and 6 of the glucose formed from [1-^{14}C]fructose as well as the presence of fructokinase and glucose-6-phosphatase in this tissue is strong evidence that the first pathway is responsible for the conversion. The operation of the other routes to any significant extent was not indicated by our experiments.

The nearly equal degree of labeling of carbon 1 and 6 of the glucose formed from [1-^{14}C]fructose indicates either that triosephosphate isomerase is very active in the intestine and allows a complete equilibration of carbon between the two triose phosphates, or that the glyceraldehyde formed from fructose-1-phosphate is not converted to glucose. That the latter is not the case is shown by the conversion of [6-^{14}C]fructose to labeled glucose.

As previously shown in the case of fructokinase[27], the enzymic equipment of the intestinal mucosa seems to vary from one species to the other. It appears possible that the inability of the rat intestine to convert fructose to glucose is related to the absence of glucose-6-phosphatase in this tissue. If this is true, the fact that glucose-6-phosphatase could not be detected in human intestine might indicate that fructose is not converted to glucose in the course of digestive absorption in man.

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PYRIDINE NUCLEOTIDES IN RAT-LIVER MITOCHONDRIA

I. THE DEMONSTRATION OF A NEW FORM OF DIPHOSPHOPYRIDINE NUCLEOTIDE AND OF TRIPHOSPHOPYRIDINE NUCLEOTIDE

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SUMMARY

1. In addition to DPN and DPNH, rat-liver mitochondria contain a diphosphopyridine nucleotide compound, which does not react with alcohol dehydrogenase.

2. DPN is liberated from this compound by incubation of the liver mitochondria with phosphate, ADP, or dinitrophenol, but not with ATP.

3. The compound was found together with DPNH in an alkali extract of fresh mitochondria. DPN was formed from both this compound and DPNH, when the extract was added to liver mitochondria, depleted of their pyridine nucleotides by incubation with phosphate in the absence of nicotinamide.

4. The new compound has the properties expected of DPN $\sim$ I, the postulated intermediate of oxidative phosphorylation.

5. A similar compound of triphosphopyridine nucleotide was also detected.

INTRODUCTION

In connexion with studies to determine whether mitochondrial isocitric dehydrogenase is a DPN- or a TPN-linked enzyme[1], it was necessary completely to deplete the mitochondria of their endogenous pyridine nucleotides. In the course of an investigation of the conversion of the reduced pyridine nucleotides to their oxidized form, which precedes the release of the oxidized form and its subsequent destruction, a new form of diphosphopyridine nucleotide was found. This was revealed by the increase in DPN reacting with alcohol dehydrogenase, over and above that accounted for by oxidation of DPNH to DPN, after incubation of mitochondria with phosphate, ADP or 2,4-dinitrophenol but not with ATP. A similar form of triphosphopyridine nucleotide was revealed by the increase in TPN reacting with TPN-specific isocitric dehydrogenase, over and above that accounted for by oxidation of TPNH to TPN. The new form of diphosphopyridine nucleotide has the properties expected of the compound DPN $\sim$ I

Abbreviations: DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide; ADP, ATP, adenosine di- and triphosphate; GSH, GSSG, glutathione and oxidized glutathione; Tris, tris(hydroxymethyl)aminomethane; fp, flavoprotein; P_i, inorganic phosphate.

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which has been postulated as an energy-rich intermediate of oxidative phosphorylation[2–4].

The interconversions of the various forms of diphosphopyridine nucleotide and of triphosphopyridine nucleotide including two transfer reactions between the new forms of TPN and DPN will be reported in a later paper. A preliminary report of some of this work has already appeared[5].

METHODS

Preparations and materials

Rat-liver mitochondria. Rat-liver mitochondria were prepared by the method of Hogeboom[6], following the procedure described by Myers and Slater[7]. Depleted rat-liver mitochondria were prepared by incubation in 0.05 *M* sucrose, 40 m*M* phosphate for 45 min (*cf.* ref. [1]).

Enzyme preparations. Yeast alcohol dehydrogenase (analytical grade) was obtained from Boehringer. *TPN-specific isocitric dehydrogenase* was prepared from pig heart up to step 3 of the method of Ochoa[8]. *TPNH-specific GSSG reductase* was prepared from peas by the method of Kaplan *et al*[9]. The purified enzyme used in this work did not oxidize DPNH.

Other preparations. The *ADP* used, obtained from Sigma Chemical Coy., contained 92 % (w/w) ADP (analysed enzymically[10]) and 2.2 moles inorg. P/100 moles ADP. *DPN* and *TPN* were obtained from Sigma Chemical Coy. and assayed for purity by yeast alcohol dehydrogenase and ethanol, and by TPN-specific isocitric dehydrogenase and isocitrate, respectively. Both were about 90–95 % pure. *DPNH* was prepared enzymically from DPN using yeast alcohol dehydrogenase and ethanol. *TPNH* was obtained from Sigma Chemical Coy., or prepared by reaction with stoichiometric amounts of TPN and D-isocitrate in the presence of TPN-specific isocitric dehydrogenase. It was assayed with GSSG and GSSG reductase. D-*Isocitric acid* was prepared from dimethyl-isocitric lactone. *GSSG* was prepared by oxidation of GSH (Boehringer, 93 % pure) by I_2.

Analytical methods

Inorganic phosphate was determined as described by Myers and Slater[7]. *Protein* was determined by the biuret method as described by Cleland and Slater[11].

Determination of pyridine nucleotides in rat-liver mitochondria

The pyridine nucleotides, DPN, TPN, DPNH and TPNH, were determined by specific enzymic fluorimetric methods applied to acid and alkali extracts of mitochondria as described by Ciotti and Kaplan[12] and Astrachan and Jacobson[13]. Fluorimetric determination of the oxidized DPN and TPN was carried out by a modification[12] of the methyl ethyl ketone method of Carpenter and Kodicek[14]. Fluorescence was measured on a Cohen[15] fluorimeter equipped with a nickel oxide glass filter in the incident beam, and a Euphor-glass filter between the solution and the photoelectric cell.

The oxidized pyridine nucleotides were determined in the extracts of mitochondria obtained by adding 0.20 ml 40 % trichloroacetic acid to 1.25 ml of a suspension containing 0.25 ml of the mitochondrial preparation. After centrifugation, the supernatant was completely decanted and neutralized with 3.5 *N* NaOH, using bromothymol blue as external indicator; 0.12 ml 3.5 *N* NaOH was usually required.

The reduced pyridine nucleotides were determined in alkali extracts of mitochondria. 1.4 ml 0.12 *M* Na_2CO_3[*] was added to a small homogenizing tube, corked, and placed in a boiling-water bath for 4 min. The mitochondrial aliquot (0.25 ml) was then quickly added to the homogenizing tube, the suspension homogenized with a Teflon plunger in the bath and left for 3–4 min. The sample was then completely transferred to a centrifuge tube, left at 0° for 10–20 min and centrifuged at low speed. The supernatant was completely decanted and then very carefully neutralized in the cold with 6 *N* HCl. Usually 2 drops were sufficient to bring the pH to 7.5–7.8. It is important that during the neutralization the pH does not go below 7.0 for even a very short period, since the reduced pyridine nucleotides are labile below this pH[**].

To make the analyses specific for the oxidized and reduced forms of the pyridine nucleotide, specific enzymes were used. Two tubes were prepared for each determination; in one, trichloroacetic acid was added before the enzyme and in the other the enzymic reaction was completed before the addition of trichloroacetic acid.

(a) *DPN*. The reaction mixture contained 0.4 ml 0.25 *M* Tris, pH 10.1, 0.05 ml 95% ethanol, and various aliquots of a DPN solution or of the neutralized trichloroacetic acid extract of the mitochondria in a total vol. of 0.80 ml. An amount of alcohol dehydrogenase (0.05 ml of 20- to 40-fold diluted enzyme) sufficient completely to reduce DPN under these conditions, without reacting with TPN, was added and the reaction stopped with 0.15 ml 40% trichloroacetic acid after 15 min at room temperature (22°). In the control tube trichloroacetic acid was added at zero time.

(b) *TPN*. The reaction mixture contained 0.4 ml 0.25 *M* Tris, pH 7.2, 0.02 ml D-isocitric acid (3.0 μmoles), 0.01 ml 0.15 *M* Mg^{++}, and various aliquots of a TPN solution or of the neutralized trichloroacetic acid extract of mitochondria in a total volume of 0.84 ml. TPN-specific isocitric dehydrogenase (0.01 ml) was added to start the reaction. The reaction was stopped with 0.15 ml 40% trichloroacetic acid after 15 min at room temperature. In the control tube trichloroacetic acid was added at zero time.

(c) *DPNH*. The reaction mixture contained 0.25 ml 0.12 *M* Na_2CO_3-$NaHCO_3$, pH 10.2; 0.2 ml 1 *M* K phosphate, pH 7.5; 0.05 ml 0.5 *M* acetaldehyde, and various aliquots of a DPNH solution or of the neutralized alkali extract of mitochondria in a total volume of 0.84 ml. 0.008 ml of 40-fold or 80-fold diluted alcohol dehydrogenase[***] was added and the reaction stopped with 0.15 ml 40% trichloroacetic acid after 3–5 min at room temperature. The amount of alcohol dehydrogenase used (40 units[§])

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[*] In many of the experiments described in this paper, a Na_2CO_3 solution of pH 10.2 (presumably obtained by absorption of atmospheric CO_2) was used. With this solution, alkaline destruction of DPN and TPN is not complete. If freshly prepared Na_2CO_3 is used, the oxidized nucleotides are completely destroyed.

[**] Since this neutralization must be carried out very carefully, it is preferable to use one of the two following procedures, which have been found to give comparable results. (1) 1.5 ml of the supernatant is added to 0.9 ml 0.25 *M* Tris, pH 7.3 + 0.15 ml 1 *N* HCl, which is sufficient to bring the pH to 7.3 (checked with bromothymol blue). 0.7 ml of this solution can be used directly for the DPNH determination without adding phosphate or Na_2CO_3. (2) 0.3 ml of the unneutralized extract + 0.3 ml 1 *M* phosphate, pH 7.5, are used for the DPNH determination.

[***] The alcohol dehydrogenase used in this study had been kept in the deep freeze for several months during which period it had been repeatedly thawed and refrozen. This treatment apparently resulted in the removal of a contaminating enzyme (see DISCUSSION).

[§] One unit is the amount of enzyme in a volume of 3.0 ml required to decrease the absorbancy by 0.01/min at pH 7.5, in the presence of excess DPNH and acetaldehyde.

was sufficient completely to oxidize the DPNH with negligible oxidation of TPNH. In the control tube trichloroacetic acid was added at zero time.

(*d*) *TPNH*. The reaction mixture contained 0.25 ml 0.25 *M* Tris, pH 7.4; 0.05 ml (5 μmoles) GSSG, pH 7.4, and various aliquots of a TPNH solution or of the neutralized alkali extracts of mitochondria in a total volume of 0.75 ml. 0.1 ml GSSG reductase[*] was added to start the reaction and the reaction stopped with 0.15 ml 40% trichloroacetic acid after 15 min at room temperature. In the control tube trichloroacetic acid was added at zero time.

After the completion of the various enzyme reactions, the fluorescence was developed by adding 0.2 ml methyl ethyl ketone followed by 0.6 ml 3.5 *N* NaOH. After 5 min at room temperature, 6 ml 0.4 *N* HCl was added and the tubes left to stand for a further 5 min. After checking that the pH was below 3, the tubes were placed in a boiling-water bath for 5 min, cooled and read in matched fluorimetric tubes. Known amounts of enzymically active DPN taken through the whole procedure were used to prepare a standard curve relating fluorescence intensity to amount of DPN. This curve was linear up to about 0.01 μmole DPN.

In the DPN and TPN determinations, the decrease in fluorescence brought by the enzymic reaction is equal to the amount of oxidized pyridine nucleotide present. (The reduced form is completely destroyed by treatment with trichloroacetic acid or with boiling HCl in the procedure used to develop the fluorescence.) Usually, but not always, the total fluorescence (corrected for the fluorescence of the reagents) obtained with the trichloroacetic acid extract of the mitochondria without reaction with enzymes was equivalent to the (DPN + TPN) content, as calculated from the separate analyses.

In the DPNH and TPNH determinations, the increase in fluorescence brought about by the enzymic reaction is equal to the amount of reduced pyridine nucleotide present.

The results of some tests of the reliability of these analytical methods are shown in Table I. None of the procedures were disturbed by the presence of the other three

TABLE I

TEST OF SPECIFICITY AND RECOVERIES OF THE VARIOUS PYRIDINE NUCLEOTIDES IN THE ANALYTICAL PROCEDURES

Known amounts of pyridine nucleotides were treated according to the various analytical procedures, either alone or added to freshly prepared liver mitochondria.

Pyridine nucleotide added		*Recovery (%) in analytical procedure for*			
		DPN	*DPNH*	*TPN*	*TPNH*
DPN	– alone	103	0	0	0
	– to mitochondria	97	—	—	—
DPNH	– alone	0	99	0	0
	– to mitochondria	—	98	—	—
TPN	– alone	0	0	92	0
TPNH	– alone	0	0	0	93
	– to mitochondria	—	—	—	104

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[*] Some preparations of GSSG reductase contain DPNH-oxidizing enzymes. The preparation used in these experiments did not oxidize DPNH in the presence of the alkali extract.

substances measured (care must be taken to choose an amount of alcohol dehydrogenase which completely oxidizes DPNH, without appreciably affecting TPNH. HOLZER[17] has shown that commercial preparations of yeast alcohol dehydrogenase contain as an impurity a TPNH-reacting alcohol dehydrogenase). The recoveries were quantitative within the limits of accuracy of the method. The reliability of the method is illustrated in Fig. 1.

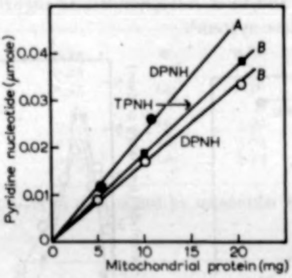

Fig. 1. Amounts of reduced pyridine nucleotides found in the alkali extract from different amounts of mitochondria. A and B represent different mitochondrial suspensions.

RESULTS

Effect of incubation of mitochondrial suspensions on concentrations of various forms of pyridine nucleotides

Figs. 2–4 describe experiments in which the mitochondria were incubated for various times at 30° with 40 m*M* phosphate (pH 7.3) in 0.05 *M* sucrose (Figs. 2A, 3 and 4) or with 1.9 m*M* ADP, 2 m*M* phosphate, 33 m*M* nicotinamide in 0.25 *M* sucrose (Fig. 2B). Fig. 2A shows that the "depletion" of diphosphopyridine nucleotide

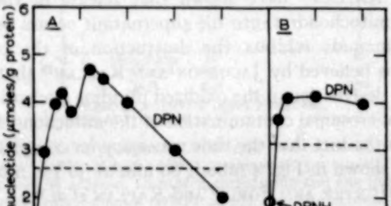

Fig. 2. Formation of DPN and disappearance of DPNH by incubation of mitochondria with phosphate or ADP. The mitochondrial suspension was gently shaken in air at 30°. A. Mitochondrial protein, 2.4 mg/ml; sucrose, 0.05 *M*; phosphate, pH 7.2, 40 m*M*. B. Mitochondrial protein, 5.6 mg/ml; sucrose, 0.25 *M*; ADP, pH 7.2, 1.9 m*M*; phosphate, pH 7.2, 2 m*M*; nicotinamide, 33 m*M*. The horizontal dotted lines show the (DPN + DPNH) at zero time.

from the mitochondria consists of at least two phases: first, the conversion of DPNH to its oxidized form, and secondly, destruction of the DPN.

The oxidation of TPNH occurred somewhat more slowly but in a similar manner (Figs. 3 and 4). The initial rates of DPN and TPN appearance shown in Fig. 4 are 1.8 and 0.8 μmoles/g protein/min, respectively. The relative rates are in good agreement with previous work which showed that the pyridine nucleotide transhydrogenase activity of depleted heart and liver mitochondria is slightly less than one-half the activity of the DPNH oxidase system[1].

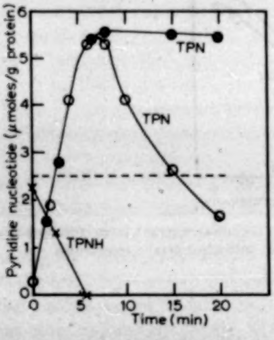

Fig. 3. Formation of TPN and disappearance of TPNH by incubation of mitochondria with phosphate. The mitochondrial suspension was gently shaken in air at 30°. O—O, ×—×, mitochondrial protein, 6.4 mg/ml; sucrose, 0.05 *M*; phosphate, pH 7.2, 40 m*M*. ●—●, same with nicotinamide, 33 m*M*. The horizontal dotted line shows the (TPN + TPNH) at zero time.

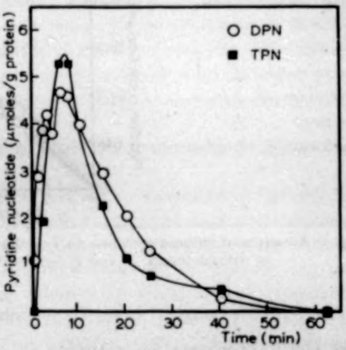

Fig. 4. Destruction of DPN and TPN by incubation of rat-liver mitochondria (6.1 mg protein/ml) in 0.05 *M* sucrose, 40 m*M* phosphate.

Kaufman and Kaplan[18] have shown that release of the oxidized pyridine nucleotide from the mitochondria into the supernatant occurs before destruction by the DPNase. Nicotinamide inhibits the destruction of the oxidized nucleotides (Figs. 2B and 3). It is believed by Jacobson and Kaplan[19] that the DPNase which is responsible for the destruction of the oxidized pyridine nucleotides is of microsomal origin and is due to microsomal contamination of the mitochondrial preparation. This may possibly explain the fact that the time necessary for complete destruction of the pyridine nucleotides shown in Fig. 4 (about 60 min at 30°) is much greater than the 15 min reported by Hunter and Ford[20] and Kaplan *et al.*[21].

The most interesting feature of Fig. 2 is that during the incubation with 40 m*M* phosphate in hypotonic medium or with 1.9 m*M* ADP, 2 m*M* phosphate in isotonic medium, the content of DPN reacting with yeast alcohol dehydrogenase increased to a value above the (DPN + DPNH) found at zero time. The extra DPN must be derived from some precursor.

The extra DPN was also formed by incubation with 2,4-dinitrophenol. Table II compares the formation of "extra" DPN, by incubation in isotonic medium with ADP and phosphate or with dinitrophenol, with that formed with 40 m*M* phosphate in hypotonic medium. Fig. 5 summarizes the result of an experiment in which the

TABLE II

FORMATION OF "EXTRA" DPN BY INCUBATION OF LIVER MITOCHONDRIA UNDER DIFFERENT CONDITIONS

The mitochondria were incubated for 10 min at room temperature with shaking in 0.05 *M* or 0.25 *M* sucrose containing 0.03 *M* nicotinamide and the additions shown. The pH was 7.4 in each case.

Addition	*Final sucrose (M)*	*Incubation time (min)*	*Diphosphopyridine nucleotide (µmoles/g protein)*			
			DPN	*DPNH*	*DPN + DPNH*	*"extra" DPN**
None	0.25	0	0.66	1.95	2.61	1.52
None	0.25	10	1.21	1.50	2.71	1.44
Phosphate (1.6 m*M*)	0.25	10	2.38	0.86	3.24	0.89
ADP (1.6 m*M*) + phosphate (1.6 m*M*)	0.25	10	3.99	0.00	3.99	0.14
Dinitrophenol (8 µ*M*)	0.25	10	3.84	0.00	3.84	0.29
Phosphate (40 m*M*)	0.05	10	4.13	—	—	—

* "Extra" DPN = total DPN as determined by incubation with 40 m*M* phosphate (4.13) — (DPN + DPNH).

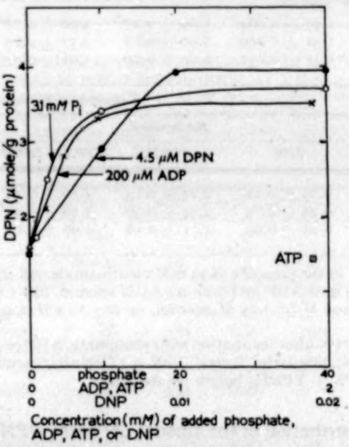

Fig. 5. Effect of concentration of phosphate, ADP and dinitrophenol on the formation of DPN from DPNH and "extra" DPN. Mitochondrial suspension (5.2 mg/ml) incubated for 5 min at 30° in the presence of 0.23 *M* sucrose, 10 m*M* nicotinamide and the indicated concentrations of phosphate, ADP, ATP or dinitrophenol (DNP) at pH 7.2. The arrows show the concentrations required for half-maximal appearance of DPN. ⊞, not-incubated mitochondria; △, mitochondria incubated with no addition.

amount of DPN formed in 5 min at 30° was studied as a function of concentration of phosphate, ADP, and 2,4-dinitrophenol. The concentrations giving half-maximal appearance of DPN in this time were 3.1 m*M* phosphate, 200 µ*M* ADP and 4.5 µ*M* dinitrophenol. In other experiments, carried out in the presence of 2 m*M* phosphate, half-maximal appearance of DPN was obtained with 70 µ*M* ADP. No DPN was formed by incubation with 1.9 m*M* ATP.

Concentrations of different forms of pyridine nucleotides in freshly prepared liver mitochondria

The concentrations of the six forms of the pyridine nucleotides in freshly prepared rat-liver mitochondria are given in Table III. Since significant differences in the DPNH and TPNH concentrations were found in mitochondria prepared in Amsterdam and Brandeis, both sets of values are given. These differences will be discussed in a following paper. The difference between the oxidized pyridine nucleotide after incubation, and the sum of oxidized and reduced nucleotide before incubation, was in every case highly significant ($P < 0.001$).

TABLE III

AVERAGE CONCENTRATION OF VARIOUS FORMS OF PYRIDINE NUCLEOTIDE IN RAT-LIVER MITOCHONDRIA

The values given (µmoles/g protein) are the means ± standard error of mean.

No. of preparations	*Prepared in*	*Diphosphopyridine nucleotide*				
		Not incubated			*Incubated**	*"extra" DPN***
		DPN	*DPNH*	*DPN + DPNH*	*DPN*	
49	Amsterdam	1.37 ± 0.064	1.69 ± 0.07	3.06 ± 0.08	4.16 ± 0.06	1.10
14	Brandeis	1.51 ± 0.066	1.26 ± 0.12	2.77 ± 0.15	4.14 ± 0.11	1.37
Total 63		1.41 ± 0.042	1.60 ± 0.06	3.01 ± 0.07	4.15 ± 0.05	1.14

No. of preparations	*Prepared in*	*Triphosphopyridine nucleotide*				
		Not incubated			*Incubated**	*"extra" TPN***
		TPN	*TPNH*	*TPN + TPNH*	*TPN*	
18	Amsterdam	0.25 ± 0.12	2.25 ± 0.15	2.50 ± 0.15	5.00 ± 0.17	2.50
13	Brandeis	0.25 ± 0.15	3.34 ± 0.30	3.59 ± 0.32	5.01 ± 0.08	1.42
Total 31		0.25 ± 0.09	2.71 ± 0.18	2.96 ± 0.18	5.00 ± 0.12	2.04

* For 7–15 min at 30° in the presence of 33 mM nicotinamide and (i) 40 mM phosphate (pH 7.2) in 0.05 M sucrose, (ii) 1.9 mM ADP (pH 7.2) in 0.05 M sucrose, (iii) 1.9 mM ADP (pH 7.2), 2 mM phosphate (pH 7.2) in 0.05 M or 0.25 M sucrose, or (iv) 10 µM 2,4-dinitrophenol in 0.05 M or 0.25 M sucrose.

** "Extra" DPN = (DPN after incubation with phosphate, ADP or dinitrophenol) — ((DPN + DPNH) before incubation). Similarly "Extra" TPN = (TPN after incubation with phosphate, ADP or dinitrophenol) — ((TPN + TPNH) before incubation).

The frequency distribution of the amount of "extra DPN" in the 63 preparations is given in Fig. 6. Only two preparations did not show any "extra" DPN.

The presence of "extra" DPN in the alkali extract

The difference in (DPN + DPNH) found at zero time and the DPN found after incubation with phosphate, ADP or dinitrophenol could be due to either (a), the "extra" DPN is stable to treatment with trichloroacetic acid and/or Na_2CO_3, but does not react with alcohol dehydrogenase, or (b), it is destroyed during the preparation of both the acid and alkali extracts.

The possible presence of the "extra" DPN in the acid and alkali extracts of mitochondria was tested by adding the neutralized extracts together with ADP and phosphate to mitochondria, previously incubated with phosphate in the absence of nicotinamide in order to deplete them of pyridine nucleotides. Using mitochondria, the amount of "extra" DPN in which had been increased by incubation with α-ketoglutarate[5], about 90 % of the "extra" DPN was recovered in the alkali extract (Table IV). None was recovered in the acid extract.

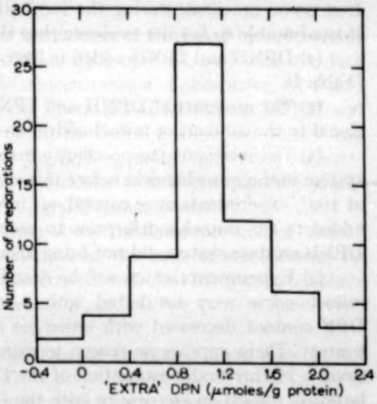

Fig. 6. Frequency distribution of the amount of "extra" DPN found in all preparations of rat-liver mitochondria analysed.

TABLE IV

Different aliquots of a mitochondrial suspension were incubated for (a) 1.5 min at 0° with no addition; (b) 1.5 min at 0° with 10 mM α-ketoglutarate; (c) 7 min at 30° with 1.9 mM ADP, 15 mM phosphate, 33 mM nicotinamide, pH 7.2. After these incubations, acid and alkali extracts were made as described in the METHODS. After measuring the DPN and DPNH contents of the neutralized extracts, aliquots of extracts (a) and (b) were added together with ADP (1.9 mM final concentration), phosphate, pH 7.2 (15 mM), and nicotinamide (33 mM) to mitochondria, previously almost depleted of their pyridine nucleotide, by incubation in 0.05 M sucrose, 40 mM phosphate, pH 7.2, for 45 min at 30°. After incubation of this mixture at 35° for 11 min, trichloroacetic acid was added and the DPN content determined.

	Expt.	*1*			*2*		
	Incubation	*(a)*	*(b)*	*(c)*	*(a)*	*(b)*	*(c)*
Acid extract							
DPN (μmoles/g protein)							
– before incubation with depleted mitochondria	(i)	2.89	1.62	3.90	1.76	0.97	4.23
– after incubation with depleted mitochondria	(ii)	2.83*	—	—	—	0.93*	—
Alkali extract							
DPNH (μmoles/g protein)	(iii)	0.59	0.16	—	1.69	1.46	—
DPN (μmoles/g protein)							
– before incubation with depleted mitochondria	(iv)	0.64	0.42	—	—	0.16	—
– after incubation with depleted mitochondria	(v)	1.38	2.52	—	—	3.18	—
– formed by incubation with depleted mitochondria	(vi)	0.74	2.10	—	—	3.02	—
– accounted for by DPNH	(vii)	0.59	0.16	—	—	1.46	—
– derived from precursor	(viii)	0.15	1.94	—	—	1.56	—
"Extra" DPN (μmoles/g protein)							
– calculated**	(ix)	0.42	2.12	—	0.76	1.80	—
(viii)/(ix) × 100		36	91	—	—	87	—

* Corrected for DPN in depleted mitochondria.

** (i) in incubation (c) *minus* [(i) + (iii)] in incubations (a) or (b), respectively.

DISCUSSION

The fact that the total DPN found after incubating the mitochondria exceeded the (DPN + DPNH) before incubation could be explained in two ways: (a) the analytical method underestimates the DPNH content of fresh mitochondria; (b) DPN is liberated from some precursor during the incubation. Before discussing the latter possibility it is advisable to list the evidence that the analytical procedure was sound.

(1) DPNH and TPNH added to liver mitochondria were recovered quantitatively (Table I).

(2) The amounts of DPNH and TPNH found in the alkali extract were proportional to the amount of mitochondria used (Fig. 1).

(3) To investigate the possibility that DPNH was oxidized by the DPNH oxidase system in the mitochondria before this enzyme system was inactivated in the Na_2CO_3 at 100°, experiments were carried out in which 1.8 m*M* Amytal or 2 m*M* KCN were added to the mitochondria prior to preparing the extracts. These inhibitors of the DPNH oxidase system did not bring about any increase in the DPNH content.

(4) Experiments which will be described in a following paper showed that when mitochondria were incubated with α-ketoglutarate, succinate or glutamate, the DPN content decreased with either no change or a slight decrease in the DPNH content. There appears no reason to expect that addition of these substrates would cause a further under-estimation of the DPNH content. The addition of β-hydroxybutyrate caused an increase in both the DPN and DPNH contents so that (DPN + DPNH) became almost equal to the DPN liberated by incubation with phosphate and ADP. There is no reason to expect that addition of β-hydroxybutyrate could improve the estimation of the DPNH.

(5) It will also be shown in a following paper that incubation of liver mitochondria with 0.8 m*M* Amytal and ADP causes the disappearance of DPNH with little change in DPN. Again, there is no reason to expect that this concentration of Amytal would cause a further under-estimation of the DPNH content. As already mentioned, a higher concentration of Amytal (1.8 m*M*) had no effect on the DPNH content.

(6) The alkali extract of mitochondria was found to contain a substance, not DPNH, which yielded DPN on incubation with mitochondria previously depleted of their own pyridine nucleotide (Table IV).

It is concluded, therefore, that the extra DPN formed by incubation of mitochondria with phosphate, ADP or dinitrophenol is due to liberation of DPN from some precursor. Although the nature or the function of this precursor is not yet established, the type of substance which causes the liberation of DPN suggests that it is connected with oxidative phosphorylation. In fact, it has the properties expected of a diphosphopyridine nucleotide compound DPN ∼ I which has been postulated as an energy-rich intermediate of oxidative phosphorylation[3,4,22], undergoing the following reactions:

$$\mathrm{DPN}\sim\mathrm{I} + \mathrm{X} \rightleftharpoons \mathrm{X}\sim\mathrm{I} + \mathrm{DPN} \qquad (1)$$

$$\mathrm{X}\sim\mathrm{I} + \mathrm{P_i} \rightleftharpoons \mathrm{X}\sim\mathrm{P} + \mathrm{I} \qquad (2)$$

$$\mathrm{X}\sim\mathrm{P} + \mathrm{ADP} \rightleftharpoons \mathrm{X} + \mathrm{ATP} \qquad (3)$$

$$\mathrm{X}\sim\mathrm{I} + \mathrm{H_2O} \xrightarrow{[(\text{dinitrophenol})]} \mathrm{X} + \mathrm{I} \qquad (4)$$

Since the passage of electrons in TPN-linked systems proceeds through a transhydrogenation reaction between TPNH and DPN to form TPN and DPNH, followed by the DPNH oxidase reaction[21,1], a similar TPN precursor was not expected. However, Fig. 3 shows the presence in liver mitochondria of a compound which liberates TPN during incubation. The significance of this compound is discussed in a following paper.

Reactions (1)–(4) are consistent with the breakdown of DPN $\sim$ I brought about by incubation with inorganic phosphate, ADP or dinitrophenol, and with the fact that ATP does not cause this breakdown. The concentrations of phosphate, ADP and dinitrophenol found to give half the maximum liberation of DPN from DPNH and "extra" DPN are comparable with those found by Chance and Connelly[23] and Chance and Hollunger[24] to be necessary for half-maximum oxidation of intramitochondrial DPNH, measured spectrophotometrically (1 mM phosphate[23]; 56 μM ADP, in the presence of 10 mM phosphate[23]; 3.0 μM dinitrophenol[24]).

The finding that ADP itself is able to bring about the decomposition of "extra" DPN without the addition of P_i can be explained on the basis of reactions (1)–(4) by the fact that the mitochondrial preparations contain sufficient inorganic phosphate for the reaction. Our preparations contain about 5 μmoles P_i/g protein, more than the total of "extra" DPN + "extra" TPN.

The fact that relatively high concentrations of inorganic phosphate in the absence of ADP cause the appearance of DPN is probably not due to the presence of sufficient X in the mitochondria, but is rather due to an "uncoupling" action of phosphate, *i.e.* incubation with phosphate causes the hydrolysis of X $\sim$ P or X $\sim$ I.

The ratios of the total triphosphopyridine nucleotide to diphosphopyridine nucleotide and of TPNH to TPN given in Table III agree closely with the values reported by Glock and McLean[25]. The total DPN content agrees with the values reported by Holton *et al.*[26] after incubation with ADP. Although most other workers (*e.g. cf.* refs. [25,27]) have reported DPNH to DPN ratios of 0.5, the higher ratio in Table III agrees with the conclusions of Chance and Williams[4] that tightly coupled mitochondria have high DPNH/DPN ratios.

The only complete study of the DPN, DPNH, TPN and TPNH content of liver mitochondria (expressed as μmoles/g protein) reported in the literature is that of Jacobson and Kaplan[27] who found DPN, 1.22; DPNH, 0.54; TPN, 0.4; TPNH, 3.92. The ratio (TPN + TPNH)/(DPN + DPNH) differs quite markedly from the values given in this paper and from those of Glock and McLean[25]. The TPNH content is higher and the DPNH value lower than our values. These differences may possibly be due to there being more "extra" DPN and less "extra" TPN in the mitochondrial preparations used by Jacobson and Kaplan. A significant difference in the concentrations of the reduced pyridine nucleotides between mitochondria prepared in different laboratories is shown in Table III. These differences are reflected in the amounts of "extra" pyridine nucleotides found.

In its stability to alkali and instability to acid, "extra" DPN resembles DPNH. However, unlike the latter it does not yield DPN on reaction with acetaldehyde* and alcohol dehydrogenase. The properties of this "extra" DPN thus bear some resemblance to those of addition compounds of DPN which are substituted on the 4-carbon atom[28-31]. The possible nature of DPN ~ I will be further discussed in a following paper.

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* Recently[16] it has been found that commercial preparations of alcohol dehydrogenase (both Boehringer and Worthington) contain an enzyme which catalyses the liberation of DPN from "extra" DPN in the presence of acetaldehyde. This enzyme, which is possibly an aldehyde dehydrogenase, disappeared on storage (during which the enzyme preparation was repeatedly thawed and refrozen). A small amount (30–40 units) of the fresh preparation is sufficient to oxidize all the DPNH in 1 min, with negligible effect on the "extra" DPN. (This was ascertained by comparison with measurements made with malic dehydrogenase + oxaloacetate, which reacts only with DPNH). A larger amount (150–200 units) reacts with nearly all the "extra" DPN in 5 min. Extracts of *Clostridium kluyverii* catalyse the liberation of both DPN from "extra" DPN, and of TPN from "extra" TPN, in the presence of acetaldehyde.

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THE COMPOSITION OF THE KETOGLUTARATE DEHYDROGENASE COMPLEX

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SUMMARY

Ketoglutarate dehydrogenase has been isolated from pig heart as a soluble complex of enzymes, containing protein bound thiamin pyrophosphate, lipoic acid and FAD in stoichiometric amounts. The flavin constituent of the complex has been shown to be lipoyl dehydrogenase. The complex was resolved into two fractions, one colourless, the other yellow, by fractionation in the presence of 2.5 *M* urea. The colourless fraction, which contains all of the protein-bound thiamin pyrophosphate and lipoic acid of the complex, was found to catalyse the oxidation of ketoglutarate when ferricyanide was used as electron acceptor. The yellow fraction was shown to be identical with lipoyl dehydrogenase (diaphorase). Neither fraction alone was capable of carrying out DPN⁺-linked ketoglutarate oxidation, this activity was reconstituted on mixing the two fractions. From chemical analysis, and the stoichiometry of the reconstitution of DPN⁺-linked ketoglutarate oxidation, the unit molecular weight of the ketoglutarate dehydrogenase complex was calculated to be about 260,000.

INTRODUCTION

The enzymic oxidation of α-ketoglutarate, like that of pyruvate, has been shown by work in the laboratories of GUNSALUS[1], REED[2] and SANADI[3] to be a complicated series of reactions involving the acylation of enzyme bound lipoic acid, transfer of the acyl group to CoASH, and reoxidation of the resulting reduced lipoic acid with DPN⁺.

$$\text{KG} + \text{enz lip S}_2 \xrightarrow{\text{TPP}} \text{CO}_2 + \text{S — succinyl lipoyl enz.} \qquad \text{(I)}$$

$$\text{S — succinyl lipoyl enz.} + \text{CoASH} \longrightarrow \text{enz lip (SH)}_2 + \text{Succinyl CoA.} \qquad \text{(II)}$$

$$\text{enz lip (SH)}_2 + \text{DPN}^+ \rightleftharpoons \text{enz lip S}_2 + \text{DPNH} + \text{H}^+ \qquad \text{(III)}$$

$$\text{overall: KG} + \text{CoASH} + \text{DPN}^+ \longrightarrow \text{succinyl CoA} + \text{DPNH} + \text{CO}_2 + \text{H}^+ \qquad \text{(IV)}$$

The work of SANADI, LITTLEFIELD AND BOCK[4] demonstrated that the enzymes responsible for the preceding reactions are associated in a soluble complex in pig heart together with the protein-bound cofactors, lipoic acid and thiamin pyrophos-

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The following abbreviations are used: DPN⁺ and DPNH, the oxidized and reduced forms of diphosphopyridine nucleotide; FAD and FADH$_2$, oxidized and reduced flavin adenine dinucleotide; lip S$_2$ and lip (SH)$_2$, oxidized and reduced lipoic acid; CoASH, coenzyme A; TPP, thiamin pyrophosphate; DCPIP, 2,6-dichlorophenol indophenol; KG, α-ketoglutarate; SU, succinate.

phate. No separation of the constituent enzymes could be obtained during purification. In this respect pig heart ketoglutarate dehydrogenase appeared to differ from the pyruvate and ketoglutarate dehydrogenases of *Escherichia coli* which were easily separated into two fractions, fraction A, precipitating at relatively low $(NH_4)_2SO_4$ concentrations, and fraction B, precipitating at higher concentrations of $(NH_4)_2SO_4$[5,6]. While complete resolution was not obtained, fraction A was rich in the enzymes catalysing reactions (I) and (II) above, and fraction B was rich in lipoyl dehydrogenase (reaction (III)). Recent reports[7,8] show that the bacterial enzymes also exist in the form of soluble complexes. Following the demonstration that diaphorase was a powerful lipoyl dehydrogenase[9,10], it was of considerable interest to determine whether the lipoyl dehydrogenase associated with ketoglutarate oxidation was the same as diaphorase, as this would provide direct evidence for the physiological role of the enzyme. Accordingly ketoglutarate dehydrogenase was purified by the method of SANADI *et al.*[4]. The resultant enzyme was amber coloured, and could be purified considerably further by fractionation on a calcium phosphate gel column, to yield a bright yellow and highly fluorescent preparation. This preparation had 2–3 times higher specific activity than that reported by SANADI, and the flavoprotein present was shown to be diaphorase (lipoyl dehydrogenase) by several methods, including resolution of the complex into two fractions, similar to the fractions A and B of the bacterial enzyme systems[6].

MATERIALS AND METHODS

DPN^+, DPNH (92 %) and TPP (85 %) were obtained from the Sigma Chemical Co., CoASH from Pabst Laboratories and the Sigma Chemical Co. DL-lipoic acid was a gift from Dr. I. C. GUNSALUS and DL-lipoamide a gift from Dr. L. J. REED. Commercial α-ketoglutaric acid was recrystallized from ethyl propionate by the method of PRICE[11]. Other chemicals used were of analytical grade.

Lipoic acid analyses: Lipoic acid was assayed in biological materials after 6 *N* HCl hydrolysis by the method of GUNSALUS AND RAZZELL[12]. The lipoic acid-deficient cells of *Streptococcus faecalis*, strain 10 Cl, were freeze dried, and kindly grown by Dr. S. R. ELSDEN from a culture provided by Dr. I. C. GUNSALUS.

Thiamin assays: Thiamin pyrophosphate was assayed spectrofluorimetrically after conversion to thiamin by seminal acid phosphatase by the thiochrome method of BURCH *et al.*[13]. The fluorescence of the thiochrome was excited with light of 370 mμ (isolated by a grating monochromator from a xenon arc). The thiochrome fluorescence was isolated from a trace of flavin fluorescence present in some samples by a combination of filters; Corning UVIOL and Chance O.V. 1. The identity of the fluorescence with that obtained from authentic TPP (or thiamin) subjected to the same method was confirmed by the identity of the fluorescence excitation spectra as defined by WEBER AND TEALE[14].

KG dehydrogenase assays: The overall oxidation of KG shown in reaction (IV) was followed spectrophotometrically at 340 mμ, in a total volume of 1 ml at 25°. Each cell (1-cm light path) contained the following constituents; phosphate pH 7.4, 60 μmoles; CoASH, 0.045 μmole; cysteine HCl, 3 μmoles; DPN^+, 0.3 μmole; KG, 1 μmole. The reaction was begun by the addition of enzyme. Unit activity is defined as that amount of enzyme producing an O.D. change of 1.0/min.

$K_3Fe(CN)_6$-linked KG dehydrogenase assays: The reaction

$$KG + 2\ Fe(CN)_6^{\equiv} \longrightarrow SU + CO_2 + 2\ Fe(CN)_6^{\equiv\!-} + 2\ H^+$$

was studied at 25° at a variety of pH values. Spectrophotometer cells contained the following constituents in a final volume of 3 ml, $K_3Fe(CN)_6$ 2 μmoles, phosphate buffer 150 μmoles, bovine serum albumin 2 mg, ketoglutarate 10 μmoles. Reaction was begun by the addition of enzyme and readings taken every 15 sec in order to determine the initial rate of reaction.

Lipoyl dehydrogenase assays: The oxidations of DPNH employing $K_3Fe(CN)_6$, lipoic acid and lipoamide as acceptors were carried out at 25° as described previously[10].

Preparation of pig heart particles: The particulate preparation from which the ketoglutarate dehydrogenase was extracted, was prepared by the method previously described[10].

Preparation of calcium phosphate gel – cellulose columns: The larger columns used in the purification of the KG dehydrogenase were prepared as described previously[10]. The smaller columns used in the urea resolution were prepared with 60 ml of the same mixture, to give a column of about 15 cm length, 1 cm diameter. The smaller columns were washed with 0.05 *M* phosphate pH 7.6 + 2.5 *M* urea and equilibrated in a cold room before use.

RESULTS

Purification of ketoglutarate dehydrogenase

A pig heart particulate preparation was extracted at pH 7–7.2 by freezing and thawing and fractionated with ammonium acetate according to the method of SANADI *et al.*[4]. The resultant amber-coloured enzyme had a specific activity of 4.35 μmoles KG oxidized/min/mg at 25° under the experimental conditions given in METHODS (variation 3.78 to 5.15 μmoles/min/mg in eight preparations). This activity is very similar to that given by SANADI *et al.*[4]. Considerable purification was then effected by fractionation on a calcium phosphate gel column. The enzyme as obtained above was dialysed for 2 h *vs* 0.1 *M* phosphate pH 7.6 and applied to a calcium phosphate gel column which had previously been washed with 0.1 *M* phosphate pH 7.6. After adsorption of the enzyme, washing with this buffer was continued, resulting in the elution of a reddish brown protein, as well as colourless protein, and leaving a bright yellow band at the top of the column. This band was then eluted with 0.1 *M* phosphate pH 7.6 + 4% (w/v) $(NH_4)_2SO_4$, and fractionated with $(NH_4)_2SO_4$ into 2 fractions; 0–0.36 saturation, and 0.36–0.70 saturation. The 0–0.36 fraction contains the KG dehydrogenase complex, the 0.36–0.70 fraction contains uncomplexed lipoyl dehydrogenase. (The complex precipitates sharply between 0.29–0.36 saturation; as no precipitate is obtained below 0.29 saturation this broader fraction is collected.) Table I summarises the results of a typical preparation.

Properties of the ketoglutarate dehydrogenase complex

The ketoglutarate dehydrogenase obtained is bright yellow and highly fluorescent, and has a specific activity of 14.9 μmoles KG oxidized/min/mg under the standard assay conditions given under METHODS. That the yellow colour and fluorescence are due to lipoyl dehydrogenase in a complex of enzymes is shown in several

TABLE I

SUMMARY OF PURIFICATION PROCEDURE FOR THE ISOLATION OF KETOGLUTARATE DEHYDROGENASE

Procedure	*Volume (ml)*	*Protein mg/ml*	*Activity units/ml*	*Specific activity units/mg*	*Total units*
pH 7.2 extract of particle preparation from 1500 g washed pig heart mince	255	13.8	55	3.98	14,000
Precipitate obtained by fractionation with ammonium acetate between 37–77 g %	15.2	30	885	29.5	13,400
Fractionated on a column of calcium phosphate-cellulose (see text) Collected yellow fraction	40	5.3	282	53	11,300
Fractionated with $(NH_4)_2SO_4$ Fraction precipitating between 0–0.36 saturation	5	23.2	2160	93	10,800

ways, which will be detailed in this and the following sections. Evidence for association of the lipoyl dehydrogenase with other proteins comes first of all from a comparison of the salting out characteristics of ketoglutarate dehydrogenase and lipoyl dehydrogenase. Ketoglutarate dehydrogenase is precipitated out of solution between 0.29 and 0.36 saturation with $(NH_4)_2SO_4$; uncomplexed lipoyl dehydrogenase is precipitated between 0.55 and 0.70 saturation[10]. The yellow colour of the ketoglutarate dehydrogenase is not due to contamination with uncomplexed lipoyl dehydrogenase, since repeated fractionation with $(NH_4)_2SO_4$ between 0.29 and 0.36 saturation fails to remove the yellow colour. Secondly in agreement with the results of SANADI *et al.*[4] ketoglutarate dehydrogenase has been found to have a high sedimentation constant. (In several determinations with a Spinco Analytical Ultracentrifuge a value of $S_{20,w}$ (extrapolated to zero protein concentration) of 30 S was found.) In contrast lipoyl dehydrogenase has a low sedimentation constant, 5.4 S[10,15]. That the yellow colour of the ketoglutarate dehydrogenase is in the form of a complex is further supported by the observation that on ultracentrifugation the yellow colour is clearly associated with this fast sedimenting material. Fig. 1 shows the Schlieren patterns obtained on ultracentrifugation of the enzyme.

Spectral comparison of ketoglutarate dehydrogenase and lipoyl dehydrogenase

Fig. 2 shows for comparison the absorption spectra of the ketoglutarate dehydrogenase complex and isolated lipoyl dehydrogenase. The relatively higher absorption of the ketoglutarate dehydrogenase at lower wavelengths may be due to two causes. Firstly, solutions of the enzyme, while appearing perfectly clear when examined with direct light, appear to scatter light appreciably when viewed with indirect light. No such phenomenon is found with lipoyl dehydrogenase. Secondly, as shown in a later section, ketoglutarate dehydrogenase contains protein-bound lipoic acid wheras lipoyl dehydrogenase does not. This would be expected to lead to a greater absorption in the 300–400 $m\mu$ region, though not as great as the observed difference.

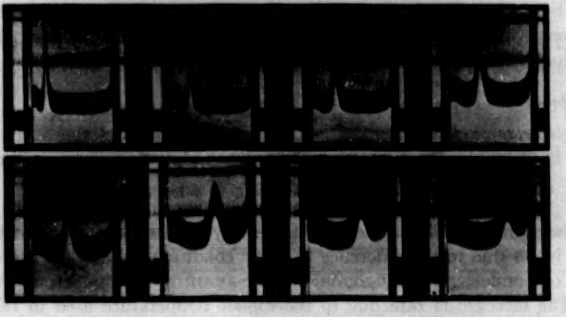


Fig. 1. Sedimentation patterns of ketoglutarate dehydrogenase (specific activity 93) obtained in the Spinco analytical ultracentrifuge. The enzyme was in 0.1 M phosphate pH 7.6, average temperature 12.1°. The total duration of the centrifugation (50,740 rev./min) was 40 min. Protein concentration, 7.7 mg/ml.

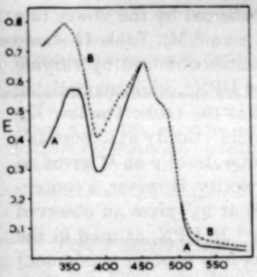


Fig. 2. Comparison of the absorption spectra of diaphorase (A) and ketoglutarate dehydrogenase (B) in 0.05 M phosphate pH 7.4. The absorptions were adjusted (by calculation) to give the same value at 455 mμ. Readings were taken at 5 mμ intervals with a Beckman model DU spectrophotometer.

Involvement of the ketoglutarate dehydrogenase flavin in ketoglutarate oxidation

The reversible reduction of the ketoglutarate dehydrogenase flavin in full accord with reactions (I–III) can be shown readily under anaerobic conditions. Fig. 3 summarises the results of several experiments of this sort. As expected from reactions I and II, the enzyme flavin is reduced only with a mixture of ketoglutarate and CoASH, neither alone causing reduction. Once reduced in this way, the flavin is reoxidized either by the addition of DPN⁺ or lipoic acid (or lipoic acid derivatives). In accordance with the involvement of protein bound reduced lipoic acid in the reduction of the flavin by ketoglutarate and CoASH, this reduction is completely

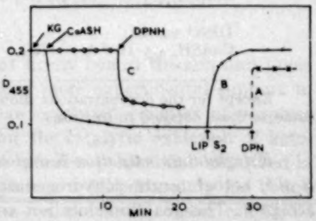


Fig. 3. The anaerobic reduction and reoxidation of flavin in the ketoglutarate dehydrogenase complex. Three anaerobic cells were used containing 0.06 M phosphate pH 7.4, and 10 mg KG dehydrogenase in a final volume of 3 ml. In each cell reduction was attempted by the addition of 0.15 μmole CoASH (reduced with cysteine) and 2 μmoles KG. Reoxidation was obtained in cell (A) on the addition of 2 μmoles DPN and in cell (B) 4 μmoles lip S_2. Cell (C) contained sodium arsenite to a final concentration of 10^{-3} M. This completely prevented reduction by KG and CoASH, but did not prevent reduction by the addition of 1 μmole DPNH. (Actually the incubation with KG and CoASH was continued for 90 min (without any change in O.D.) before the addition of DPNH; the time scale has been foreshortened here for the sake of convenience). Temperature, 4°.

inhibited by $10^{-3}\ M$ sodium arsenite. Also in accord with the flavin of ketoglutarate dehydrogenase being lipoyl dehydrogenase, reduction of the flavin is obtained by the addition of DPNH (reaction (III)); this reduction is not inhibited by arsenite.

Enzymic properties of the α-ketoglutarate dehydrogenase complex

Ketoglutarate oxidation coupled to DPN reduction: Under the assay conditions described in METHODS, the rate of ketoglutarate oxidation in the overall reaction:

$$\text{KG} + \text{DPN}^+ + \text{CoASH} \longrightarrow \text{Succinyl CoA} + \text{DPNH} + \text{H}^+ + \text{CO}_2$$

is 14.9 µmoles/min/mg. This rate is considerably higher than reported by SANADI *et al.* and is not due to the difference in assay conditions. When the above reaction is carried out under the assay conditions of SANADI *et al.*[8] the rate is 15.4 µmoles/min/mg. The increase in rate due to the higher temperature used in the assay of SANADI *et al.*[8] (30° instead of 25°) is apparently almost balanced by the slower rate due to the lower DPN concentration ($10^{-4}\ M$ instead of $3 \cdot 10^{-4}\ M$). Table II shows the Michaelis constants and extrapolated maximum velocities obtained by varying separately the concentrations of ketoglutarate, CoASH and DPN+, while maintaining the other substrate concentrations constant as described in the table. The low K_m for CoASH permits the measurement of essentially maximum velocity at almost any concentration of CoASH, and a concentration of ketoglutarate of 0.01 M gives an observed velocity of 98 % of the extrapolated maximum velocity. However, a concentration of DPN of $1 \cdot 10^{-4}\ M$ (employed by SANADI *et al.*[8]) at 25° gives an observed velocity only 68 % the extrapolated maximum. (At $3 \cdot 10^{-4}\ M$ DPN, as used in the standard assay described in this paper, the observed velocity is 87 % of the maximum.) Thus the maximum velocity for infinite concentration of all substrates should approximate that found for DPN, *i.e.* 17.1 µmoles/min/mg.

TABLE II

MICHAELIS CONSTANTS AND MAXIMUM VELOCITIES OF THE REACTION

$$\text{KG} + \text{CoASH} + \text{DPN}^+ \longrightarrow \text{Succinyl CoA} + \text{CO}_2 + \text{DPNH} + \text{H}^+$$

*Variable**	*Extrapolated maximum velocity µmoles DPN+ reduced/min/mg*	K_m
CoASH varied; DPN+, $3 \cdot 10^{-4}\ M$; KG, $10^{-2}\ M$	14.9	$< 10^{-7}\ M$
KG varied; CoASH, $4.5 \cdot 10^{-5}\ M$; DPN+, $6 \cdot 10^{-4}\ M$	16.0	$1.3 \cdot 10^{-5}\ M$
DPN+ varied; CoASH, $4.5 \cdot 10^{-5}\ M$; KG, $10^{-2}\ M$	17.1	$4.5 \cdot 10^{-5}\ M$

* Except for the concentrations shown in this column, the experimental conditions were the same as those detailed in METHODS.

Ketoglutarate oxidation linked to ferricyanide reduction: As reported by SANADI *et al.*[4], ketoglutarate dehydrogenase also oxidises ketoglutarate using $K_3Fe(CN)_6$ as acceptor. This reaction does not require CoASH or DPN, and is not inhibited by $10^{-3}\ M$ sodium arsenite, as is the DPN-linked reaction discussed previously. The reaction was shown to be:

$$\mathrm{KG} + 2\ \mathrm{Fe(CN)}_6^{\equiv} \longrightarrow \mathrm{Succinate} + \mathrm{CO}_2 + 2\mathrm{H}^+ + 2\ \mathrm{Fe(CN)}_6^{≣}$$

The experimental evidence for this stoichiometry is given in Table III. The pH optimum for this reaction was found to be pH 6.4. The K_m for ferricyanide, and the extrapolated maximum velocity, at 25° and pH 6.5 were respectively $2 \cdot 10^{-4}\,M$; 4.1 µmoles KG oxidized/min/mg.

TABLE III

STOICHIOMETRY OF $\mathrm{K_3Fe(CN)_6}$-LINKED OXIDATION OF KETOGLUTARATE

The reduction of $\mathrm{K_3Fe(CN)_6}$ was carried out at pH 6.8 as described in METHODS. In the second experiment, succinate was measured by the micromethod employing phenazine methosulphate-catalysed reduction of cytochrome c[20].

Experiment	*µmoles KG present*	*µmoles $K_3Fe(CN)_6$ reduced*	*µmoles succinate produced*	*Conclusion*
Enzyme reacted with limiting amounts KG in presence of excess $\mathrm{K_3Fe(CN)_6}$	0.5 1.0	1.0 2.0	— —	$\mathrm{KG} + 2\ \mathrm{Fe(CN)}_6^{\equiv} \rightarrow$
Enzyme reacted with excess KG and limiting amount of $\mathrm{K_3Fe(CN)_6}$. When $\mathrm{K_3Fe(CN)_6}$ was fully reduced, enzyme was destroyed by heating at 100° for 1 min, and succinate estimated	5 5	1.0 2.0	0.455 0.995	$\mathrm{KG} + 2\ \mathrm{Fe(CN)}_6^{\equiv}$ ↓ $\mathrm{SU} + 2\ \mathrm{Fe(CN)}_6^{≣}$ $(+ \mathrm{CO}_2 + 2\mathrm{H}^+)$

Lipoyl dehydrogenase activity: As well as reacting with the protein bound lipoic acid, the lipoyl dehydrogenase of the ketoglutarate dehydrogenase complex also reacts with added lipoic acid and derivatives[8, 16]. It also exhibits the ferricyanide linked DPNH dehydrogenase (diaphorase) activity of lipoyl dehydrogenase. The rates of reaction with these substrates, per mole of flavin, are identical with those of uncomplexed lipoyl dehydrogenase. Furthermore the Michaelis constants, at least under the conditions tested, are very similar. Thus it would appear that the complexing of lipoyl dehydrogenase in ketoglutarate dehydrogenase does not hinder its reaction with added lipoic acid derivatives or with pyridine nucleotides:

$$\mathrm{DPNH} + \mathrm{H}^+ + \mathrm{lip\ S}_2 \rightleftharpoons \mathrm{DPN}^+ + \mathrm{lip(SH)}_2$$

A comparison of the experimental values with ketoglutarate dehydrogenase and isolated lipoyl dehydrogenase is given in Table IV.

Bound cofactors in ketoglutarate dehydrogenase

The early work of SANADI *et al.*[4] showed that firmly bound thiamin and lipoic acid were present in their preparations, and added more experimental support to the concept of ketoglutarate dehydrogenase as an organized complex of enzymes provided with some of the necessary cofactors for the catalytic oxidation of ketoglutarate. This finding is confirmed with the more purified preparation described in this paper. Table V shows the analysis of ketoglutarate dehydrogenase for bound thiamin pyrophosphate and lipoic acid, and gives as well the flavin content calculated from the yellow colour of the enzyme. It is seen that there are approximately equimolar amounts of thiamin pyrophosphate, lipoic acid and flavin, suggesting that the

TABLE IV

COMPARISON OF KINETIC CONSTANTS OF DIAPHORASE AND KETOGLUTARATE DEHYDROGENASE

All assays were at 25°, conditions as described previously[10], *i.e.* pH 5.9 with lipoic acid as acceptor, pH 6.5 with lipoamide and pH 4.8 with $K_3Fe(CN)_6$. V_{max} values are expressed as turnover numbers, *i.e.* as moles of DPNH oxidized/min/mole of enzyme flavin. The flavin concentration was calculated assuming a value of E_{455} of $1.13 \cdot 10^4$ cm²/mmole in both diaphorase and ketoglutarate dehydrogenase.

Acceptor	DL-lipoic acid		DL-lipoamide		$K_3Fe(CN)_6$	
Enzyme	V_{max}	Km mM	V_{max}	Km mM	V_{max}	Km mM
Diaphorase	1000	2	80,000	5	8000	0.27
Ketoglutarate dehydrogenase	1000	5	80,000	5.5	8400	0.22

TABLE V

BOUND COFACTORS OF KETOGLUTARATE DEHYDROGENASE

(+) lipoic acid and thiamin pyrophosphate were assayed as described in METHODS. Flavin was estimated from its O.D. at 455 mµ assuming E_{455} of $1.13 \cdot 10^4$ cm²/mmole.

Enzyme	(+) Lipoic acid µmoles/g	Thiamin pyrophosphate µmoles/g	Flavin µmoles/g
Ketoglutarate dehydrogenase complex	4.5	3.1	4.1
As II (colourless fraction obtained in urea resolution)	5.3	4.5	0
As III (yellow fraction obtained in urea resolution)	0.1	0	12.8
Diaphorase	0	0	13.3

constituent enzymes of the complex are present in stoichiometric amounts, presumably linked in a rather special way for the most efficient oxidation of ketoglutarate. The average value of 3.9 µmoles/g protein indicates a minimum molecular weight of 257,000 for the ketoglutarate dehydrogenase complex. This is considerably smaller than the value of 2,000,000 estimated by SANADI *et al.*[4] and confirmed here by the high sedimentation constant. Thus it would appear that ketoglutarate dehydrogenase as isolated is a polymer of a unit complex of enzymes; that about eight such unit complexes form one giant molecule. From the resolution of the complex with urea (detailed in the following section) it would appear that the forces holding the complex together are probably hydrogen bonds.

Resolution of the ketoglutarate dehydrogenase complex with urea

Ketoglutarate dehydrogenase can be resolved into two distinct protein fractions by treatment with urea. When the enzyme was incubated at 0° with different concentrations of urea, it was found even with the highest concentration tested (5.5 *M* urea) that the lipoyl dehydrogenase activity was not affected even after two days. This concentration of urea was sufficient, however, to destroy both DPN⁺- and $K_3Fe(CN)_6$-linked ketoglutarate oxidations almost instantaneously. Examination in the analytical ultracentrifuge showed that such treatment had produced considerable polydispersity, suggesting a dissociation of the ketoglutarate complex. Incubation with 1.1 M urea produced no inactivation of ketoglutarate oxidation even after 24 h; in 2.5 M urea full activity was maintained for at least 3 h and only half the activity was destroyed in 24 h; in 3.7 M urea 60 % of the activity was lost in 2 h and complete inactivation obtained before 24 h. This instability of the enzyme responsible for the initial stages of ketoglutarate oxidation places a fairly limited restriction on the concentration of urea which can be employed in attempting to separate the components of the complex. In practice fractionation in the presence of 2.5 M urea was found satisfactory; good resolution was obtained and comparatively little inactivation was obtained during the time taken to accomplish the fractionation. A typical resolution is described below.

A dialysed solution of ketoglutarate dehydrogenase was made 0.05 M with respect to phosphate pH 7.6 and 2.5 M with respect to urea and applied to a calcium phosphate gel column (see METHODS) which had previously been washed with 0.05 M phosphate pH 7.6 + 2.5 M urea. After adsorption of the enzyme the column was washed with the same buffer–urea solution. A colourless protein fraction was eluted in this way, leaving a yellow, fluorescent band still at the top of the column. This band was then eluted with 0.075 M phosphate pH 7.6 + 2.5 M urea + 4 % (w/v) $(NH_4)_2SO_4$. The elution pattern obtained is shown in Fig. 4. The colourless fraction, which contains the urea-unstable enzyme(s) was precipitated with $(NH_4)_2SO_4$ (to 0.45 saturation) as soon as possible after elution, the precipitate redissolved in 0.1 M phosphate pH 7.6 and then reprecipitated with $(NH_4)_2SO_4$ in order to dilute out the urea as rapidly as possible. The yellow fraction was concentrated by precipitation with $(NH_4)_2SO_4$ between 0.45 and 0.7 saturation. The $(NH_4)_2SO_4$ precipitates of both fractions were dissolved in 0.05 M phosphate pH 7.4. Neither fraction alone was able to catalyse the DPN⁺-linked oxidation shown by the original enzyme; however on mixing the two fractions this activity was reconstituted (Fig. 5). As with the un-

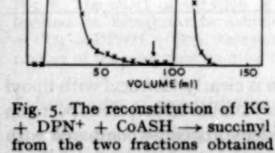
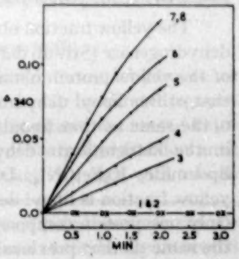


Fig. 4. The resolution of the KG dehydrogenase complex with 2.5 M urea employing a column of calcium phosphate gel suspended on cellulose. Elution of the first (colourless) fraction was obtained with 0.05 M phosphate pH 7.6 + 2.5 M urea. At the point indicated by the arrow the yellow band was still at the top of the column. It was then eluted with 0.075 M phosphate pH 7.6 + 2.5 M urea + 4 % (w/v) $(NH_4)_2SO_4$.

Fig. 5. The reconstitution of KG dehydrogenase activity (KG + DPN⁺ + CoASH → succinyl CoA + CO_2 + DPNH + H⁺) from the two fractions obtained by urea resolution. Assays were carried out at 25° as detailed in METHODS. Curve 1 shows the lack of effect of 2.12 µg of the colourless fraction in the absence of added yellow fraction. Curve 2 shows the lack of effect of 48 µg of the yellow fraction in the absence of added colourless fraction. Curves 3–8 show the effect of mixing 2.12 µg of the colourless fraction with varying amounts of the yellow fraction. The amounts of yellow fraction used were; Curve 3, 0.194 µg; curve 4, 0.292 µg; curve 5, 0.584 µg; curve 6, 1.94 µg; curves 7 and 8, 5.84 µg and 17.5 µg.



resolved enzyme, this reconstituted activity is inhibited completely by 10^{-3} *M* arsenite.

The colourless fraction contains all of the protein-bound thiamin pyrophosphate and lipoic acid of the ketoglutarate dehydrogenase complex (Table V). As would be expected from the flavoprotein nature of lipoyl dehydrogenase, the colourless fraction is completely devoid of lipoyl dehydrogenase activity. This fraction is however capable of ketoglutarate oxidation when ferricyanide is used as acceptor. This reaction is identical with the reaction catalysed by the intact complex; it has the same stoichiometry and the same pH optimum (pH 6.4). Also the specific activities are quite similar. The maximal rate with the intact ketoglutarate dehydrogenase is 4.1 µmoles ketoglutarate oxidized/min/mg at pH 6.5; the corresponding value with the colourless fraction is 6.0. This difference probably does not represent a real increase in activity on dissociation of the complex, but is merely a reflection of lower protein content/unit activity caused by the separation of the constituents of the complex (*cf.* Table VI).

TABLE VI

TURNOVER NUMBERS AT 25° OF REACTIONS CATALYSED BY THE KETOGLUTARATE DEHYDROGENASE COMPLEX AND ITS CONSTITUENT ENZYMES

The turnover numbers were calculated from the values previously quoted, assuming molecular weights of 260,000 for the whole complex, 180,000 for the colourless fraction (As II), and 80,000 for diaphorase (As III).

Reaction	*pH*	*Max turnover number*		
		Whole complex	*As II*	*As III*
KG + DPN^+ + CoASH ⟶ Succ CoA + DPNH + H^+ + CO_2	7.4	4,400	0	0
KG + 2 $Fe(CN)_6^{\equiv}$ ⟶ SU + CO_2 + $2H^+$ + 2 $Fe(CN)_6^{\equiv}$	6.5	1,060	1,080	0
H^+ + DPNH + lipoic ⇌ DPN^+ + reduced lipoic	5.9	1,000	0	1,000
H^+ + DPNH + lipoamide ⇌ DPN^+ + reduced lipoamide	6.5	80,000	0	80,000
DPNH + $2Fe(CN)_6^{\equiv}$ ⟶ DPN + $2Fe(CN)_6^{\equiv}$	4.8	8,400	0	8,000

Properties of the yellow fraction

The yellow fraction obtained by the urea resolution is clearly identical with lipoyl dehydrogenase (Straub diaphorase). Fig. 6 shows the very similar absorption spectrum of the yellow protein obtained from the ketoglutarate dehydrogenase compared with that of free lipoyl dehydrogenase[10]. The lipoyl dehydrogenase activity/mole of flavin is the same as that found either with free lipoyl dehydrogenase or with the enzyme in the ketoglutarate dehydrogenase complex (DPNH oxidation with lipoic acid, lipoamide, $K_3Fe(CN)_6$, DCPIP as acceptors). Further convincing proof that the yellow fraction is lipoyl dehydrogenase comes from ultra centrifugal analysis (Fig. 7). Only one protein component was observed, with a sedimentation constant of 5.4 S, the same as that previously reported for lipoyl dehydrogenase[15,10].

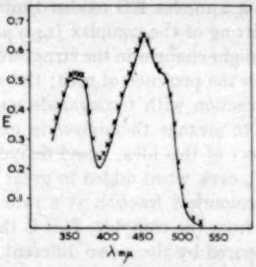


Fig. 6. Comparison of the visible spectrum of diaphorase[10] and the yellow fraction obtained by urea resolution of the KG dehydrogenase complex (see text). O.D. readings were taken at 5 mμ intervals with a Unicam SP 500 spectrophotometer. The solid line shows the spectrum of a solution of diaphorase in 0.05 *M* phosphate pH 7.4, protein concentration 4.3 mg/ml. The crosses show the O.D. under the same conditions of a 4.7 mg/ml solution of the yellow fraction obtained by urea resolution from ketoglutarate dehydrogenase.

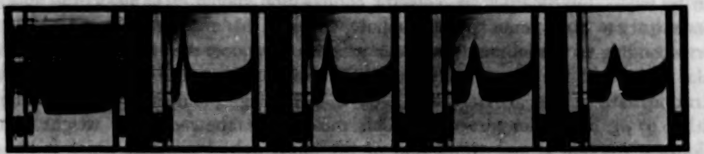


Fig. 7. The sedimentation of the yellow fraction from urea resolution in the Spinco analytical ultracentrifuge. The enzyme (4.7 mg/ml) was in 0.1 *M* phosphate pH 7.6; average temperature 17.5°; rotor speed 50,740 rev./min. Photographs were taken at 16 min intervals.

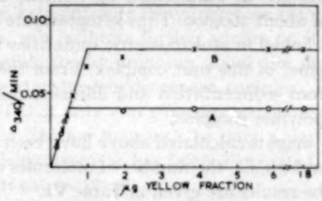


Fig. 8. The effect of titration of the colourless fraction from urea resolution with the yellow fraction on ketoglutarate dehydrogenase activity (KG + DPN$^+$ + CoASH $\longrightarrow$ succinyl CoA + CO_2 + DPNH + H$^+$). Assays were carried out at 25° as detailed under METHODS. In curve A 1.06 μg of the colourless fraction was used; in curve B 2.12 μg of the colourless fraction was used.

Stoichiometry of reconstitution of ketoglutarate dehydrogenase activity

Fig. 8 shows that in the reconstitution of the overall ketoglutarate dehydrogenase activity obtained by mixing the two fractions, each fraction may be titrated against the other. Thus if the amount of colourless fraction is kept constant, the rate of ketoglutarate oxidation is directly proportional to the amount of the yellow fraction until saturation is obtained, when there is no further increase in activity even with a large increase in the yellow fraction.

From these results the specific activity of the reconstituted enzyme can be calculated. At the most favourable, *i.e.* when the one fraction is theoretically saturated with the other the activity is 4.2 µmoles KG oxidized/min/mg. This is considerably less than the value before splitting of the complex (14.9 µmoles/min/mg). The lower value obtained may be due to slight changes in the structure of either or both fractions as a result of the separation in the presence of urea; the slightly modified enzymes, though unimpaired in their reaction with ferricyanide and externally added lipoyl derivatives, may not be able to arrange themselves in exactly the same way as in the natural complex. In support of this idea, lipoyl dehydrogenase prepared by the method previously described[10], even when added in great excess, functions in ketoglutarate oxidation with the colourless fraction at a rate only 0.1–0.25 that of the lipoyl dehydrogenase isolated from the complex. This is the only difference that has been found in the enzyme prepared by these two different procedures.

Estimates of unit molecular weight

From the sedimentation constant of 30 S a very high molecular weight is indicated. SANADI *et al.*[4] have given an estimate of 2,000,000 from their data. From two quite distinct sets of results reported here, estimates of minimum molecular weight can be made. Firstly from the cofactor composition shown in Table V, the average concentration of thiamin pyrophosphate, lipoic acid and FAD is 3.9 µmoles/g, corresponding to a minimum molecular weight of 257,000. An estimate of the molecular weight of the enzymically functional complex can also be made from the titration data of Fig. 8. Here it is seen that 2.12 µg colourless fraction combines with 0.97 µg lipoyl dehydrogenase. This means that the molecular weight of the colourless fraction is 2.20 times that of the lipoyl dehydrogenase, which is known to be about 80,000[15]. Thus the molecular weight of the colourless fraction would be about 180,000, and of the whole complex, about 260,000. The values obtained by these two different methods are in remarkable agreement, and give an average minimal molecular weight of about 260,000. Thus ketoglutarate dehydrogenase is not only a complex of enzymes linked in stoichiometric quantities together with tightly bound cofactors, but a polymer of this unit complex. From the molecular weight of SANADI *et al.*[4] calculated from sedimentation and diffusion data, there would be 8 such unit complexes/giant polymer complex.

The minimal molecular weights calculated above have been used to convert rates of reaction into turnover numbers, *i.e.* the number of molecules of substrate oxidized/min/molecule of enzyme. The results are given in Table VI.

DISCUSSION

The results show conclusively that the lipoyl dehydrogenase of the ketoglutarate dehydrogenase complex is a flavoprotein, and identical with the lipoyl dehydrogenase already described[10] (STRAUB diaphorase[15,17]). Thus there can be little doubt that the physiological function of diaphorase is as the lipoyl dehydrogenase of keto acid oxidation. Its function in ketoglutarate oxidation can be represented as in Fig. 9. It should be noted that while this enzyme can readily catalyse the reversible reaction,

$$\text{DPNH} + \text{H}^+ + \text{lip S}_2 \rightleftharpoons \text{DPN}^+ + \text{lip(SH)}_2$$

where lip S_2 applies to a number of lipoyl derivatives (including that bound in the ketoglutarate dehydrogenase complex), its normal function in oxidative metabolism

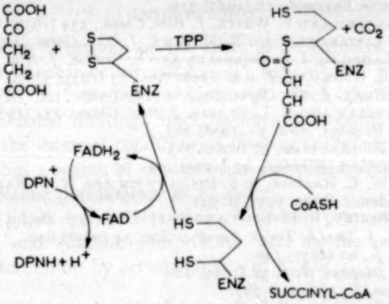


Fig. 9. Scheme of ketoglutarate oxidation by the KG dehydrogenase complex.

would be to reduce DPN+, rather than to oxidize DPNH, the function previously ascribed to STRAUB diaphorase[18]. The DPNH formed is presumably reoxidized *in vivo* by way of the respiratory chain of enzymes. The pattern of electron and hydrogen transfer is thus much more complex with α-ketoglutarate than with any other of the tricarboxylic acid cycle intermediates:

$$\underset{\text{(lipoyl dehydrogenase)}}{\text{KG} \longrightarrow \text{Lip S}_2 \longrightarrow \text{FAD}} \longrightarrow \underset{\text{(DPNH dehydrogenase)}}{\text{DPN}^+ \longrightarrow \text{flavin}} \longrightarrow \text{cytochrome system}$$

It is somewhat of a paradox that thiamin pyrophosphate, the first of the four vitamin cofactors recognized to be required in keto acid oxidation[19], is the only one whose function is not reasonably well understood. It is hoped that its presence in stoichiometric amounts in the ketoglutarate dehydrogenase complex, and its localization in the colourless fraction, will facilitate further studies on its function.

It is not yet possible to say anything about the number of different enzymes involved in the ketoglutarate dehydrogenase complex. The results of the urea resolution would suggest that only two enzymes are involved, but it must be pointed out that the colourless fraction may well be only a partly resolved complex, from which all the lipoyl dehydrogenase has been dissociated, but which may consist of any number of constituent enzymes.

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INFLUENCE OF INORGANIC PHOSPHATE IN THE FORMATION OF PHOSPHATASES BY *ESCHERICHIA COLI*

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SUMMARY

Both acid and alkaline phosphatase have been demonstrated in strains of *E. coli*. Bacterial acid phosphatase (pH optimum 4–5) displays a fairly high specificity for the hexosephosphates, while the alkaline enzyme (pH optimum 8.5–9.5) hydrolyzes all the phosphomonoesters tested.

The kinetics of formation of both enzymes have been studied and it has been shown that alkaline phosphatase in measurable amount is only formed when Pi becomes limiting in the medium, at which point the enzyme is formed in substantial amount at a maximum rate.

The implications of the findings with respect to mechanisms of controlling enzyme formation are discussed.

The following abbreviations are used: Pi (inorganic phosphate); Tris (trishydroxymethylaminomethane); NPP (*p*-nitrophenylphosphate); NP (*p*-nitrophenol); bis-NPP (bis-*p*-nitrophenylphosphate).

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INTRODUCTION

During the course of investigations on the effect of depletion of various nutrients on the level of certain enzymes in cultures of *E. coli**, high alkaline phosphatase activities were observed in cells harvested from media in which the inorganic phosphate concentration had become limiting. Since significant amounts of this enzyme are formed only under the unusual condition of limiting inorganic phosphate, perhaps it is not surprising that a search of the literature revealed but one short reference to the occurrence of alkaline phosphatase in *E. coli*[1].

In the present paper we are reporting on certain properties of two phosphomonoesterases, acid and alkaline phosphatase, and on the influence of inorganic phosphate on their formation by certain strains of *E. coli*.

METHODS

Bacterial strains: Most of the experiments to be reported were carried out using *E. coli* strain W although other strains (ML and K12) were shown to behave similarly.

Cultural conditions: Organisms were cultivated on minimal media of the following basal composition per l: (a) Basal Medium A; K_2HPO_4, 7 g; KH_2PO_4, 3 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $(NH_4)_2SO_4$, 1 g and sodium citrate, 0.5 g. The pH was adjusted to 7.0 with NaOH. (b) Basal Medium A-P: This medium was identical with medium A except that it contained 0.1 *M* Tris instead of phosphate. The pH was adjusted to 7.4 with HCl.

The sterile basal medium was distributed in Erlenmeyer flasks and glucose or sodium lactate, as carbon source, was added before inoculation. Care was taken to avoid adding Pi with the inoculum in experiments involving medium A-P, either by washing with Pi-free medium or by use of very small inocula. Cultures were incubated at 37°, with mechanical shaking to insure adequate aeration.

Bacterial growth: Growth was followed at 490 mμ in the Beckman spectrophotometer. It was found that an O.D. of 1 was equivalent to about 0.7 mg bacteria (dry wt.)/ml and approximately $5 \cdot 10^9$ cells/ml in cultures during exponential growth.

*Chemostat experiments***: Under conditions where Pi was limiting medium A-P contained either 0.1 % glucose or 0.2 % sodium lactate as carbon source and 50 μmoles of K_2HPO_4/l. When the carbon source was limiting, the Pi concentration was increased to 170 μmoles and the lactate concentration dropped to 0.02 %.

In these experiments the inoculum was grown overnight in minimal medium A-P containing 50 mμmoles/ml of K_2HPO_4 and 0.01 % sodium lactate; under these conditions the growth was limited by the energy source at O.D. 0.050. The following day enough lactate was added to bring its concentration to 0.2 %, growth resumed and when the O.D. reached 0.090 (see Fig. 1, curve A), the culture was transferred to the growth tube of the chemostat and feeding was started immediately. Flow rates were established for each experiment.

Determination of enzyme activity: Acid and alkaline phosphatase activities were determined in cell suspensions or cell-free extracts. 2-ml samples of culture were placed in centrifuge tubes containing 0.2 ml 1:1000 merthiolate in order to block instantly further enzyme formation. The killed cells were centrifuged at 2° and washed with chilled 0.1 *M* Tris buffer at pH 7.4. The washed cells were resuspended in exactly 2 ml of buffer. This suspension was either used directly or was first treated by shaking with 0.02 ml toluene/ml at 37° for 30 min. As will be shown below, treatment with merthiolate or with toluene or even complete disruption had no effect on enzyme activity.

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* Preliminary experiments carried out in the Dept. of Cellular Physiology, Pasteur Institute, Paris.

** Chemostat experiments have been carried out in the Department of Bacteriology, Harvard Medical School. I thank Dr. B. D. DAVIS for permitting me to use this facility.

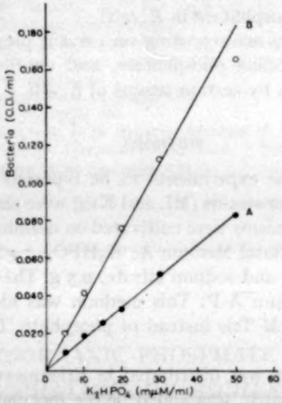

Fig. 1. Yield of bacteria as a function of Pi concentration in the medium. The cultures were grown in medium A-P with K_2HPO_4 added in increasing concentrations. Curve A shows the O.D. at cessation of the initial rapid logarithmic growth. Curve B shows the O.D. at the stationary phase (18–24 h).

Both acid and alkaline phosphatases were measured by a modification of the methods of OHMORI[2] and of BESSEY *et al.*[3]. The *p*-nitrophenol (NP) liberated from *p*-nitrophenylphosphate (Sigma 104) (NPP) at 37° was measured at 420 mμ and an alkaline pH. The following procedures were adopted.

Acid phosphatase: To 3 ml of 0.2 *M* acetate buffer at pH 4.0 and 37° are added 1 ml 0.04 *M* NPP and 1 ml of bacterial suspension (intact, toluenized or sonicated). The reaction mixture is maintained at 37° in a water bath. 1-ml samples are withdrawn at intervals and immediately added to 1 ml of 1 *M* Tris buffer, pH 8.5, containing 0.4 *M* phosphate. A high Pi concentration is essential at this point to inhibit any alkaline phosphatase activity which may be present. The amount of NP liberated is then measured at 420 mμ in the Beckman spectrophotometer. The amount of NP liberated after at least 4 successive intervals is plotted against time and the activity calculated from the slope of the resulting linear curve.

Alkaline phosphatase: 1 ml 1 *M* Tris, pH 8.8 and 0.5 ml of bacterial suspension are placed in a Beckman cuvette and brought to 37° in the chamber of the spectrophotometer. After the correct temperature is reached, 0.5 ml of 0.04 *M* NPP at 37° is added with rapid mixing. The appearance of free NP is followed directly at 420 mμ. The unit of enzyme activity is defined as that amount of enzyme which liberates 1 mμmole of Pi (or NP) per h under the prescribed conditions.

Pi determination: In determining the activity of the phosphatases on substrates other than NPP, it was necessary to follow liberation of Pi directly. Pi was determined using the method of FISKE AND SUBBAROW[4] as modified by DRYER *et al.*[5]. At low Pi concentrations (10 mμmoles/ml or less) it was necessary to use a bioassay method. The solution to be tested was used as a phosphate source for growth of coli W wild type. After 24 h the O.D. was measured and Pi concentration read on the standard calibration curve shown in Fig. 1B.

RESULTS

Effect of Pi on growth of E. coli

Preliminary experiments were performed to test the behavior of cultures growing in media containing limited amounts of Pi. Increasing amounts of K_2HPO_4 (5–50 mμmoles/ml) were added to medium A-P using 0.3 % lactate as energy source. The initial period of exponential growth was followed by a phase of decreasing growth rate beginning abruptly when Pi became limiting. An increase in turbidity per unit bacterial dry wt. from an O.D. of 1.54/mg during exponential growth to 2.28/mg when growth finally ceased after Pi became limiting was found. In curve A of Fig. 1 is plotted the O.D. of the culture at the time Pi became limiting as a function of Pi concentration. Curve B shows the O.D. 18–24 h later at the same Pi concentration. It is of interest to note that although the slope of curve B is 2.24 times that of curve A, the bacterial yield in terms of dry wt. increased by only 50 % over the 18–24-h period.

The effect of Pi on the rate of exponential growth was also studied but no significant change in rate of growth was observed even with Pi concentrations as low as 5 mμmoles/ml.

Demonstration of acid and alkaline phosphatase activity

Most living cells are known to contain at least two types of phosphatases, acid and alkaline. *E. coli* suspensions were therefore tested over a wide pH range using, as a buffer, mixtures of 0.1 *M* sodium acetate and 0.1 *M* Tris adjusted with 0.2 *N* HCl to pH values between 3 and 10.

Fig. 2 shows the effect of pH on phosphatase activity in two bacterial suspensions, each of which was washed twice with water and resuspended in water to approximately the same O.D. Suspension A was prepared from cells grown on excess glucose but limiting Pi. Suspension B was prepared from a culture in which glucose was the limiting factor and Pi was in excess. As seen from Fig. 2, suspension A showed two regions of phosphatase activity, a minor zone which was maximal near pH 4 and a

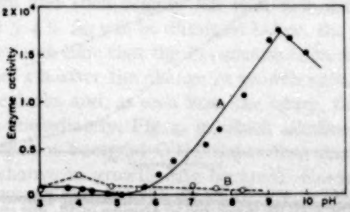

Fig. 2. Phosphatase activity as a function of pH. Enzyme activity: mμmoles of NP liberated/h/ml of cell suspension. Curve A: culture limited on Pi. Curve B: culture limited on glucose.

major region with peak activity at pH 9.3. Suspension B showed appreciable phosphatase activity only in the neighborhood of pH 4. Thus *E. coli* suspensions may show both acid and alkaline phosphatase but only cultures limited on Pi will show high activity at alkaline pH's.

Some properties of the two phosphatases

Both acid and alkaline phosphatase activities of *E. coli* are closely associated with the cells. After centrifugation only 10–15 % of the activity remains in the culture supernatant. Most of the activity is sedimented with the cells whether living or killed by merthiolate. The activity was the same whether or not the cells were intact and living or completely disrupted by sonication. The properties to be discussed below were all determined using sonic extracts from washed suspensions of cells harvested from an overnight culture grown on media containing excess carbon source and an initial concentration of 300 mμmoles/ml Pi so as to limit growth to about 1.2 O.D.

Stability

Toluenized suspensions of *E. coli* show no loss of either acid or alkaline phosphatase activity even after many days storage in the refrigerator. Even after 5 min at 100°, only 50 % of the alkaline phosphatase activity is destroyed.

pH optimum

The optimal pH found using NPP as substrate varied somewhat depending upon the nature of the buffer used. The optimum found for acid phosphatase ranged between pH 3 and pH 5; for alkaline phosphatase between 8.8 and 9.8. The same pH optima were found with sonic extracts as with whole bacterial suspensions.

Inhibitors

The effect of Pi on the activity of both acid and alkaline phosphatases has been tested at their respective pH optima. Fig. 3 shows that alkaline phosphatase is strongly inhibited by Pi and that inhibition is nearly complete in the presence of 0.1 *M* phosphate. Acid phosphatase is less affected by Pi. On the other hand the activity

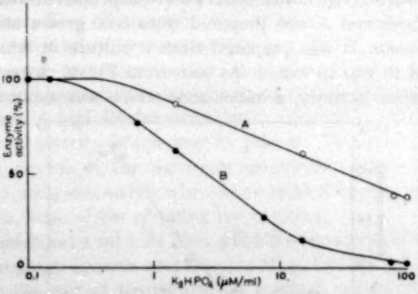

Fig. 3. Inhibition of phosphatase activity by Pi. The activities measured in presence of Pi are expressed as percentage of the activity measured in absence of Pi. For convenience the Pi concentrations have been plotted on a logarithmic scale. Curve A: activity measured at pH 4.0. Curve B: activity measured at pH 8.8.

of acid phosphatase is inhibited 85 % in the presence of 0.01 *M* sodium fluoride; this concentration is without effect on the activity of the alkaline enzyme. The behavior of the two phosphatases from *E. coli* with respect to Pi and fluoride inhibition is similar to that of the analogous enzymes from other microorganisms[6], and from animal sources[7–10].

Specificity

The activity of sonically disrupted bacterial suspensions was tested on several organic phosphate esters at pH 4 and at pH 8.8. The results summarized in Table I show that in both the acid and the alkaline range the enzyme preparation does not hydrolyze ATP and hydrolyzes bis-NPP to only slight extent (2 % or less of the NPP hydrolysis). Identical results were obtained using the cell suspensions before sonic treatment. It can be concluded that both acid and alkaline enzymes are phosphomonoesterases deprived of pyro- and di-esterase activity. While, as shown in Table I, the alkaline enzyme hydrolyzed all of the phosphomonoesters tested, the acid enzyme displayed a fairly high specificity for the hexosephosphates. Both enzymes hydrolyzed NPP readily.

TABLE I

SPECIFICITY OF ALKALINE AND ACID PHOSPHATASES

Substrate	*pH 8.8* *mμmoles Pi/h/ml*	*pH 4* *mμmoles Pi/h/ml*
NPP	27,000	690
AMP	19,300	0
β-glycerophosphate	16,100	43
Glucose 6-phosphate	18,000	560
Fructose-1,6-phosphate	17,350	645
Bis-NPP	trace	trace
ATP	0	0

Influence of Pi in the formation of alkaline phosphatase

In the experiment illustrated by Fig. 4, the kinetics of growth and alkaline phosphatase formation were followed in a culture limited on Pi. The bacteria were grown in duplicate flasks of medium A-P containing 0.2 % glucose and 200 mμmoles/ml K_2HPO_4. Fig. 4a shows the growth curves obtained for the two cultures. The growth rate remained constant at $\mu = 1.0$ (*i.e.* one division/h) until the bacterial density reached O.D. = 0.750/ml and then rapidly fell to a new rate $\mu = 0.2$ which was maintained for the next 3–4 h. As will be discussed below, the change in growth rate took place at almost the same time that the Pi concentration reaches its lowest value in the culture supernate. 1 h after the change in growth rate, 1 μmole/ml K_2HPO_4 was added to one of the flasks and, as seen from the figure, the original growth rate ($\mu = 1.0$) was resumed immediately. Fig. 4, in which alkaline phosphatase activity as ordinate is plotted against bacterial O.D., shows that enzyme formation began at the time when the change in growth rate occurred. Enzyme formation quickly ceased in the flask to which 1 μmole/ml Pi had been added and the enzyme activity in this culture remained constant thereafter. The differential rate of appearance of enzyme was high (= 106,000 units/O.D.) and remained constant for about 3 h after the change in growth rate. It may be noted at this point that the amount of acid phosphatase formed per cell is always the same and independent of Pi concentration.

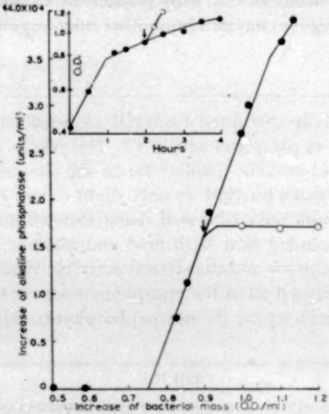

Fig. 4. Kinetics of formation of alkaline phosphatase. The main figure shows the increase of enzyme activity per ml as a function of the growth of the cultures. The insert shows the growth curve of the cultures. See text for details. ●—● culture in medium with limiting Pi. ○—○ culture after the addition of Pi. The arrow indicates the addition of 1 μmole of K_2HPO_4/ml.

Behavior of cultures grown in mixtures of organic and inorganic phosphate

In order to obtain more precise information regarding the relationship of Pi concentration to alkaline phosphatase formation, growth and enzyme synthesis were studied in cultures containing both Pi, in limited amount, and organic phosphate in excess. Curves 1 of Fig. 5 show the results of an experiment using *E. coli* W grown in medium A-P to which 50 mμmoles/ml K_2HPO_4 and 400 mμmoles/ml β-glycerophosphate had been added. Sodium lactate served as carbon source*. Curves 2 in Fig. 5 give the results in the control culture which contained only the inorganic source of phosphate. Inspection of Fig. 5 shows that in the control culture, change in growth rate, disappearance of detectable extracellular Pi and onset of a maximum rate of enzyme formation all coincided at a time indicated by the arrows. The behavior of the culture containing organic phosphate was different. A short, but reproducible diauxic lag lasting about 12–15 min is shown in curve 1 of Fig. 5A. The inorganic phosphate in the medium continued to fall during the diauxic lag. Enzyme formation began at about the time that growth was resumed and the Pi concentration had fallen to a minimum. The presence of enzyme caused a rapid release of Pi from glycerophosphate and further enzyme synthesis was suppressed, as soon as the Pi level reached 20–25 mμmoles/ml and the specific phosphatase activity had risen to ca. 5000 units/O.D. After the Pi concentration had increased to 75 mμmoles/ml, growth had reached a point where the rate of utilization of Pi exceeded the rate of its release by enzymic hydrolysis. Phosphatase synthesis was resumed a second time during the 4th hour of the experiment when the Pi concentration had again fallen to a minimum.

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* Glucose was not used as the source of carbon in this experiment since the acid produced from glycolysis might favor activity of acid phosphatase.

This experiment shows that when both organic and inorganic phosphate are available it is the inorganic Pi that is used preferentially.

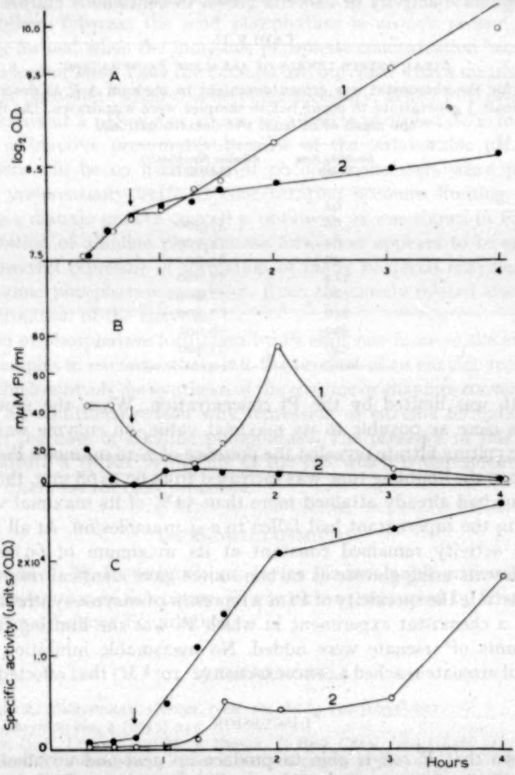

Fig. 5. Bacterial growth, extracellular Pi and alkaline phosphatase activity. The results with two cultures are shown one growing in a medium containing both inorganic and organic phosphate, and the other containing only inorganic phosphate. A, curve of growth; B, variation of Pi concentration in the cell free medium; C, alkaline phosphatase formation. Curves 1: medium containing K_2HPO_4 (50 mμmoles/ml) + β-glycerophosphate (400 mμmoles/ml). Curves 2: medium containing K_2HPO_4 (50 mμmoles/ml). The arrows indicate the onset of the diauxic lag.

Steady state levels of alkaline phosphatase in continuous cultures grown on limiting Pi

After the Pi becomes exhausted by a culture grown on limiting Pi, there is a prolonged period during which O.D. of the culture slowly increases and phosphatase is formed (see Fig. 4). During this period the ratio of bacterial mass to O.D. decreases and it is probable that the bacterial phosphorus content is also decreasing. The fact that alkaline phosphatase is formed at a constant rate during this stage implies that its differential rate of synthesis is dependent of changes in growth rate of the cells and is independent of changes in their composition. This conclusion has been verified by experiments in the chemostat. Table II shows the steady state values attained for alkaline phosphatase activity in bacteria grown in continuous culture in which the rate of growth was limited by the Pi concentration. When the growth rate was maintained as close as possible to its maximal value, no enzyme was formed and analysis of the culture filtrate revealed the presence of 7–10 mμmoles Pi/ml*. Table II shows that when the doubling time was increased from 62 to 68 min, the steady state level of enzyme had already attained more than 50 % of its maximal value and the Pi remaining in the supernatant had fallen to 2–4 mμmoles/ml. At all lower growth rates enzyme activity remained constant at its maximum of 69,000 units/O.D. Similar experiments using glucose as carbon source gave identical results.

TABLE II

STEADY STATE LEVELS OF ALKALINE PHOSPHATASE

The inoculum for the chemostat was grown overnight in medium A-P as described. Time was allowed for at least 5 generations to occur before samples were withdrawn. The figures represent the mean of at least two determinations.

Doubling time (min)	*Alkaline phosphatase (Units/O.D.)*
62	0
68	37,500
84	74,000
90	65,000
114	75,000
180	71,200
240	67,500
360	66,000
540	65,400

To demonstrate the specificity of Pi as a repressor of enzyme synthesis, Na_2HAsO_4 was tested in a chemostat experiment in which Pi was the limiting factor and increasing amounts of arsenate were added. No measurable inhibition of synthesis was noted until arsenate reached a concentration ($2 \cdot 10^{-4}$ *M*) that affected growth rate.

DISCUSSION

We have shown that *E. coli* is able to produce an acid and an alkaline phosphomonoesterase. The experiments reported concern only strain W, but both ML and K12 strains were also tested with similar results. The two phosphatases resemble analogous enzymes that have been studied in other microorganisms and in animal tissues in their pH optima as well as in their behavior toward inhibitors such as inorganic phosphate and fluoride. Neither enzyme is excreted into the culture medium; only after mechanical disruption of the cells are they obtained in the soluble fraction. All the substrates tested however, are equally well hydrolyzed by both disrupted and intact cells. Consequently these enzymes appear to be freely accessible *in vivo* to the substrates present in the surrounding medium.

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* For Pi analysis 5-ml samples were withdrawn from the chemostat, rapidly chilled to 0° and filtered through a millipore filter. The whole operation required no more than 2–3 min.

The two enzymes differ in their substrate specificity. The alkaline phosphatase appears to hydrolyze to the same extent monohexosephosphates, mononucleotides and synthetic phosphomonoesters. The specificity of the acid phosphatase seems to be confined mostly to the hexosephosphates.

The conditions for formation of the two bacterial phosphatases differ strikingly from one another. Whereas the acid phosphatase is always present, the alkaline enzyme is only formed when the inorganic phosphate concentration becomes limiting (10 mμmoles Pi/ml or less). Thus the bacteria are provided with a means of obtaining Pi from organic phosphate when this becomes necessary. When Pi is present in excess and growth occurs at a pH of 7 or above, no alkaline phosphatase is formed and the acid enzyme is inactive presumably because of the unfavorable pH. Under such conditions there will be no hydrolysis of phosphomonoesters when present and Pi will be used preferentially until its concentration becomes limiting. Under these circumstances a diauxic growth curve[11] is obtained, as was shown in Fig. 5.

The inhibition of alkaline phosphatase formation appears to be specific for Pi. Glucose, a powerful repressor of formation of many bacterial enzymes[12] is without effect on alkaline phosphatase synthesis. Even the closely related arsenate ion does not repress formation of the enzyme.

Inhibition of phosphatase formation by Pi adds one more to the ever-increasing number of examples in bacteria where it is the product of an enzyme reaction or series of reactions which controls the synthesis of the enzyme or enzymes concerned (see[12–14]).

There is an additional reason why repression of enzyme formation may be of importance in the case of alkaline phosphatase. The presence of this enzyme may actually constitute a threat to the life of the cell which is dependent on a variety of phosphate esters for its vital processes.

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A FINE-STRUCTURE GENETIC AND CHEMICAL STUDY OF THE ENZYME ALKALINE PHOSPHATASE OF *E. COLI*

I. PURIFICATION AND CHARACTERIZATION OF ALKALINE PHOSPHATASE

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SUMMARY

A procedure for purification of alkaline phosphatase from *E. coli* is described, and several physical-chemical properties of the purified enzyme are reported. When the cells are grown under the condition of phosphate deprivation, which is optimal for the synthesis of alkaline phosphatase, about 6 % of the total protein synthesized is alkaline phosphatase.

INTRODUCTION

The main physiological role of genetic material appears to be the determination of the specificity of protein molecules. This generalization is based on the many examples known of mutations which cause the loss or change of an enzymic function[1], and particularly on those cases for which there is direct evidence that a mutation causes the formation of an altered protein molecule[2-6]. Studies of genetically controlled alterations in haemoglobin by INGRAM, using the technique of comparative fingerprinting[7,8], have shown that a single mutation can result in the replacement of one amino acid at a particular position in the polypeptide chain by a different amino acid. This finding indicates that alterations in protein specificity brought about by mutations can involve changes in the amino acid sequence of the molecule. Several mechanisms have been proposed to account for the genetic control of amino acid sequences in proteins[9,10].

The aim of the present work is the development of a system with which it will be possible to do fine-structure genetic analysis[11-13] of mutations affecting a single enzyme, and at the same time to identify chemical alterations in the enzyme molecule resulting from the mutations. For this purpose the enzyme alkaline phosphatase produced by the bacterium *Escherichia coli* strain K12 was selected. The following factors influenced this choice:

1. The bacterium is suitable for genetic analysis, since crosses can be performed with large populations by pairwise mating between cells[14,15] or by transduction with phage P1[16]. It will be shown later in the course of this work that phosphatase-negative mutants can be isolated and, in crosses between these negative mutants, rare phos-

phatase-positive genetic recombinants can be detected, thereby satisfying the requiring for fine-structure mapping.

2. Recent work on the alkaline phosphatase of *E. coli* by Dr. A. M. TORRIANI[17] (who kindly informed us of her results before publication) shows that the enzymic activity in a culture can be regulated over a wide range by control of the concentration of inorganic phosphate in the growth medium. The maximal level of alkaline phosphatase activity is obtained with a medium in which the concentration of phosphate is sufficiently low so that it becomes the growth-limiting constituent. This finding suggested that under conditions of phosphate limitation the cells might produce the enzyme in high enough concentration to make the purification relatively simple.

3. Alkaline phosphatase is a reasonably stable enzyme, and its activity is easily assayed by following the color change of the substrate *p*-nitrophenyl phosphate[17].

This paper will report the methods that have been developed for purifying alkaline phosphatase from *E. coli*, and also some physical-chemical properties of the purified material.

MATERIAL AND METHODS

A prototrophic Hfr strain of *E. coli* K12 was used in all experiments. This strain was isolated as a spontaneous revertant from the Hfr strain CS101 which requires methionine for growth[18]. CS101 derives from the Hfr originally isolated by CAVALLI[19].

The composition of the growth medium used to obtain high yields of alkaline phosphatase is as follows: $8 \cdot 10^{-2}$ *M* NaCl; $2 \cdot 10^{-6}$ *M* $FeCl_3$; $2 \cdot 10^{-2}$ *M* KCl; $1.2 \cdot 10^{-1}$ *M* Tris buffer pH 7.5; $2 \cdot 10^{-2}$ *M* NH_4Cl; $1.2 \cdot 10^{-2}$ *M* glucose; $1 \cdot 10^{-3}$ *M* $MgCl_2$; $1.4 \cdot 10^{-4}$ *M* sodium glycerophosphate; $2 \cdot 10^{-4}$ *M* $CaCl_2$; 0.04 % Bacto-peptone (Difco); $5 \cdot 10^{-4}$ *M* Na_2SO_4. It was found convenient to prepare 20-l cultures in a glass carboy vigorously aerated through several sintered-glass filters. The medium was inoculated with about $2 \cdot 10^7$ cells/ml and incubated at 37° for 20 h.

For preparing cultures labeled with ^{14}C proline, the growth medium was modified by eliminating the Bacto-peptone and sodium glycerophosphate, and adding KH_2PO_4 in the required amounts. This medium will be called P medium.

Alkaline phosphatase activity was assayed by incubating the enzyme with 0.2 mg/ml of the substrate *p*-nitrophenyl phosphate (NPP) in 1.0 *M* Tris buffer pH 8.0, which is the pH optimum for the reaction. The increase in absorption at 410 mμ, resulting from the dephosphorylation of NPP to yield nitrophenol, was followed spectrophotometrically with a cell of 1-cm path length. Under these conditions the enzymic activity is linearly dependent on the enzyme concentration in the range of 0.001 to 1.0 O.D. units/min at 25°, and in this range is independent of substrate concentration when it is in excess of 0.1 mg/ml. In this assay the enzymic activity is not affected by the addition of 0.01 *M* Mg^{++} to the medium. However, if the concentration of Tris buffer is lowered to 0.01 *M*, full enzymic activity is not obtained without the addition of Mg^{++}. A Mg^{++} activation effect has generally been found with other phosphatases[20].

N,N-Diethylaminoethyl-cellulose[21] (DEAE) (obtained from Distillation Products Industries, Rochester, New York) was washed extensively with 0.1 *M* Tris buffer at pH 7.4 before use. It was packed to a height of about 10 cm under eight pounds per square-inch pressure into a chromatography tube containing a sintered-glass disk at the bottom. The packed DEAE column received a final wash with 0.01 *M* Tris buffer pH 7.4. The enzyme material, which had been dialyzed against 0.01 *M* Tris buffer pH 7.4, was applied to the column and elution was carried out with a linearly increasing concentration of salt. The eluting solution was passed through the column at a rate of about 0.5 ml/min, and was collected in separate tubes on a fraction collector.

RESULTS

Synthesis of alkaline phosphatase protein in cultures starved for phosphate

The fact that the amount of alkaline phosphatase in *E. coli* is optimal when the cells are starved for phosphate, and is markedly reduced when there is an excess of phosphate available[17], suggested that the enzyme protein might be identified as a component which is found only in cells starved for phosphate. The following experiment shows a comparison of protein fractions obtained from two cultures, one of which was starved for phosphate while the other was provided with an excess.

A log-phase culture grown in P medium containing $1.6 \cdot 10^{-3}$ *M* KH_2PO_4 (excess phosphate) was centrifuged and resuspended in P medium without added phosphate at a density of $5 \cdot 10^8$ cells/ml. The resuspended cells were divided into two cultures, A and B, with 100 ml in each; culture A remained without added phosphate and culture B was adjusted to $1.6 \cdot 10^{-3}$ *M* KH_2PO_4. The cells were aerated on a shaker at 37° for 20 min, and then 5 μC of labeled L-[^{14}C]proline (specific activity 13 μC/μmole) were added to each culture. Aeration was continued for 4 h, after which the cultures were centrifuged and the cells resuspended in 10 ml of a medium containing 0.05 *M* Tris buffer pH 7.4 and 0.02 *M* $MgSO_4$. The cells were then ruptured in a French decompression press[22] by release from a pressure of 10,000 pounds/sq. in. The presence of $MgSO_4$ in the medium serves to preserve intact the ribosomal particles during this treatment[23]. The ruptured cells were exposed to 20 μg/ml of DNAase for 20 min at 37°, and were then centrifuged at 35,000 rev./min in a Spinco head 40 for 1 h at 5°. The pellet material was discarded and the supernatants were dialyzed for 20 h at 5° against a solution containing 0.01 *M* Tris buffer at pH 7.4 and 0.001 *M* $MgSO_4$. Of the total ^{14}C activity that had initally been incorporated in the cells, 61 % was present in the dialysate of culture A and 52 % in the dialysate of culture B. At this stage there was added to the dialysate of B unlabeled carrier alkaline phosphatase from a culture which had been prepared in the same manner as culture A, except that [^{14}C]proline had not been added. The dialysates were then passed through a column of DEAE; under these conditions all of the alkaline phosphatase activity attached to the DEAE. Elutions from the column were carried out with a linear gradient of NaCl ranging from 0 to 0.14 *M*, buffered at pH 7.4 with 0.01 *M* Tris. Eluate fractions were collected and assayed for ^{14}C and alkaline phosphatase activities.

The results for cultures A and B are plotted in Figs. 1 and 2. Culture A, which was starved for phosphate and consequently produced alkaline phosphatase at an optimal rate, shows a single prominant peak of ^{14}C activity which corresponds to the peak of enzymic activity. The ^{14}C peak represents 8.4 % of the total [^{14}C]proline initially present in the cells; all of the alkaline phosphatase activity initially present in the cells is accounted for in the peak of enzymic activity. In culture B, which was provided with an excess of phosphate and consequently produced only 1/200 as much

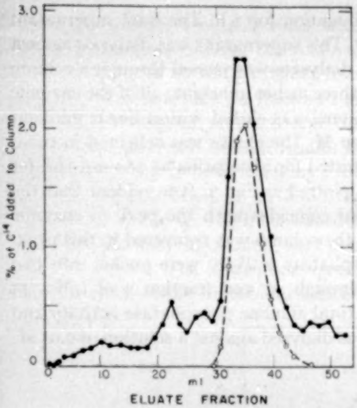

Fig. 1. Synthesis of alkaline phosphatase protein by cells grown in phosphate-limited medium. A cell extract was prepared by the procedure described in the text and adsorbed to a column of DEAE. Elution was achieved by a linear gradient of NaCl ranging from 0 to 0.14 *M* buffered at pH 7.4 with 0.01 *M* Tris. The eluate fractions were assayed for [^{14}C]proline, which was used as a specific label for the cellular protein (solid line curve), and for alkaline phosphatase enzymic activity (broken line curve).

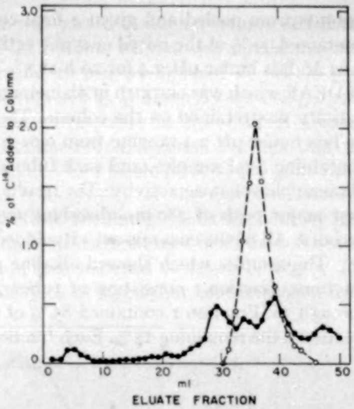

Fig. 2. Lack of synthesis of alkaline phosphatase protein by cells grown in a medium containing excess orthophosphate. The experimental procedure was the same as for Fig. 1.

alkaline phosphatase activity as culture A, there is no prominent ^{14}C peak in the region where the enzymic activity (added as unlabeled carrier) comes off the column. Within the region containing the carrier enzymic activity, 2.4 % of the ^{14}C initially present in the cells is found. This ^{14}C represents the amount of non-enzyme protein material that comes off the column along with the alkaline phosphatase. Subtracting this amount from the amount found in the enzyme peak in culture A, we obtain the result that 6 % of the proline incorporated into cellular protein, at the time the culture was producing alkaline phosphatase at an optimal rate, was alkaline phosphatase proline. Since proline constitutes 4.8 moles percent of the amino acids in alkaline phosphatase protein, which is close to the value of 4.6 moles percent reported for the total protein of *E. coli* grown in synthetic medium[32], we conclude that about 6 % of the total protein synthesized was alkaline phosphatase protein.

Larger-scale purification of the enzyme

A 20-l culture was grown in a medium containing limiting amounts of phosphate by the procedure described in the section on methods. Purification of the alkaline phosphatase was achieved as follows. The cells were centrifuged and resuspended in 100 ml of 0.1 *M* Tris buffer pH 7.4; all of the enzymic activity remained with the cells. The cells were equilibrated at 82° for 15 min (alkaline phosphatase is stable at this temperature—*cf.* section on properties of the enzyme) and then were dialyzed against 0.1 *M* Tris bufter 7.4 for 20 h at 5°. The dialysate was centrifuged at 20,000 rev./min in a Spinco head 20 for 20 min, and the pellet material was put through three successive cycles of centrifugation and resuspension in 50 ml of 0.1 *M* Tris pH 7.4. All supernatants were pooled and given a final centrifugation for 1 h. The final supernatant contained 90 % of the initial enzymic activity. This supernatant was dialyzed against 0.02 *M* Tris buffer pH 7.4 for 20 h at 5°. The dialysate was passed through a column of DEAE which was one inch in diameter and three inches in height; all of the enzymic activity was retained on the column. The enzyme was eluted with a linear gradient of Tris buffer pH 7.4 ranging from 0.02 to 0.20 *M*. The eluate was collected in tubes containing 4-ml samples, and each tube was tested for absorption at 280 mμ and for alkaline phosphatase activity. The results are plotted in Fig. 3. It is evident that the first major peak of 280 mμ-absorbing material coincides with the peak of enzymic activity. All of the enzymic activity added to the column was recovered in this peak.

The samples which showed alkaline phosphatase activity were pooled into two fractions, fraction 1 consisting of tubes 17 through 22 and fraction 2 of tubes 23 through 36. Fraction 1 contained 85 % of the total alkaline phosphatase activity and fraction 2 the remaining 15 %. Each fraction was dialyzed against a solution of 0.01 *M*.

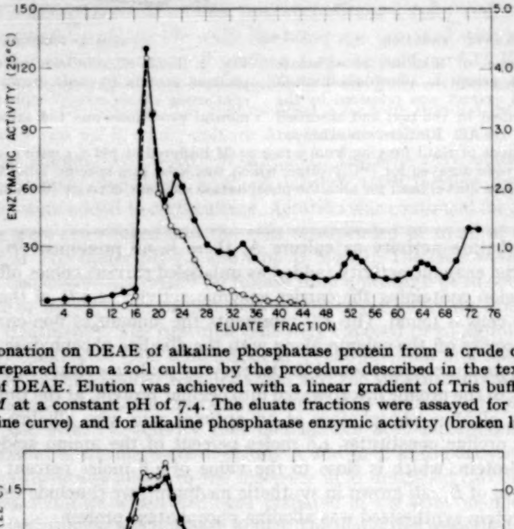

Fig. 3. Fractionation on DEAE of alkaline phosphatase protein from a crude cell extract. The extract was prepared from a 20-l culture by the procedure described in the text and adsorbed to a column of DEAE. Elution was achieved with a linear gradient of Tris buffer ranging from 0.02 to 0.20 *M* at a constant pH of 7.4. The eluate fractions were assayed for O.D. at 280 mμ (solid line curve) and for alkaline phosphatase enzymic activity (broken line curve).

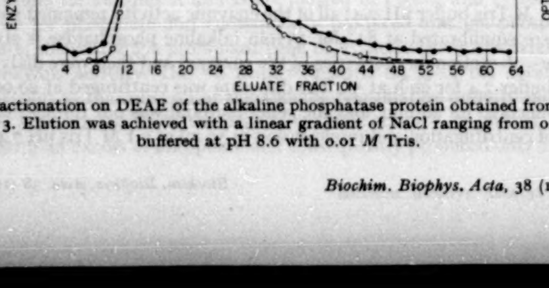

Fig. 4. Re-fractionation on DEAE of the alkaline phosphatase protein obtained from the enzyme peak in Fig. 3. Elution was achieved with a linear gradient of NaCl ranging from 0.04 to 0.10 *M* buffered at pH 8.6 with 0.01 *M* Tris.

Tris buffer pH 8.6 + 0.04 *M* NaCl, and then was passed through a column of DEAE. All of the enzymic activity was retained on the column. Elution was carried out with a linear gradient of NaCl ranging from 0.04 *M* to 0.10 *M* buffered at pH 8.6 with 0.01 *M* Tris. The results for fraction 1 are plotted in Fig. 4. There is only a single peak of 280 mμ-absorbing material and this peak corresponds to the peak of alkaline phosphatase activity. Similar results were obtained for fraction 2. The fact that the enzymic activity of both fractions 1 and 2 eluted as a single peak in the same range of NaCl concentration is an indication of homogeneity of the enzyme. Tubes 12 through 31 of Fig. 4, which contained 80 % of the alkaline phosphatase activity that had been added to the column, were pooled and this material used in all the succeeding tests for purity. The absorption spectrum of this material is shown in Fig. 5. The absence of any peak at 260 mμ indicates the lack of significant contamination from nucleic acid.

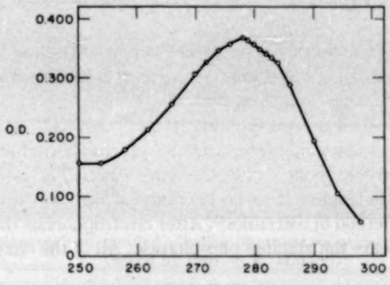

Fig. 5. Absorption spectrum of purified alkaline phosphatase protein.

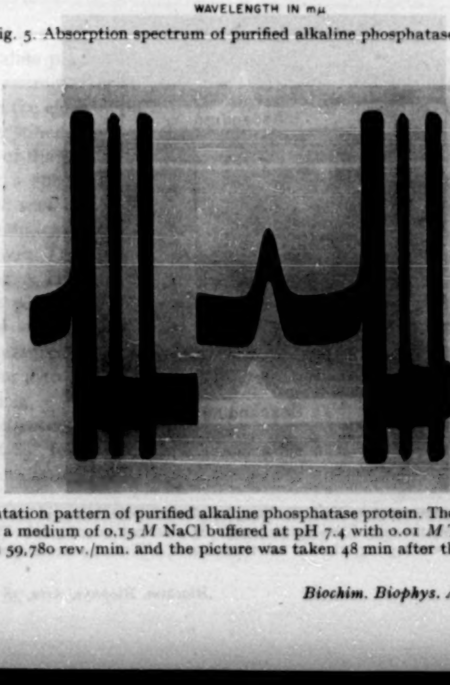

Fig. 6. Sedimentation pattern of purified alkaline phosphatase protein. The enzyme concentration was 2 mg/ml in a medium of 0.15 *M* NaCl buffered at pH 7.4 with 0.01 *M* Tris. The centrifugation speed was 59,780 rev./min. and the picture was taken 48 min after the start of the run.

Further evidence for the purity of the enzyme and some of its properties

Sedimentation constant and molecular weight: When the purified material was examined in the analytical ultracentrifuge there was a single schlieren peak which appeared symmetrical as shown in Fig. 6. The sedimentation constant, obtained at 20° in 0.15 *M* NaCl buffered at pH 7.4 with 0.01 *M* Tris, was 6.3S. This value agrees with the result reported for hog kidney phosphatase[21].

The molecular weight was determined by the ARCHIBALD method as described by EHRENBERG[25]. Using a partial specific volume of 0.73 which was estimated from a preliminary amino acid analysis of the purified enzyme, a molecular weight of 75,000 to 80,000 was calculated. Taking this molecular weight value to calculate the frictional constant f_0 of the equivalent sphere[26], and the sedimentation constant and molecular weight values to calculate the true frictional constant f, a value of f/f_0 of 1.05 is obtained. This indicates that the molecule is approximately spherical and is not greatly hydrated.

Electrophoresis: Electrophoresis was carried out in a Tiselius type cell at three different pH's, 7.6, 6.5, and 4.4, at an ionic strength of 0.10. The results from each of these runs showed only a single schlieren peak which appeared to be symmetrical, as shown in Fig. 7. The electrophoretic mobility at pH 7.6 was $-3.3 \cdot 10^{-5}$ cm²/V sec. (The mobility of swine kidney phosphatase[21] in pH 7.2 phosphate buffer of ionic strength 0.10 has been found to be $-1.8 \cdot 10^{-5}$ cm²/V sec.) The isoelectric point was estimated to be 4.5. Electrophoresis was also carried out in starch gel at pH 8.0, 4.5, and 3.6, using the method of SMITHIES[33]. After electrophoresis the gel was sprayed with NPP in order to locate the alkaline phosphatase. All of the enzymic activity was

Fig. 7. Electrophoresis pattern of purified alkaline phosphatase protein at pH 7.6. Electrophoresis was carried out in the micro-cell of the Spinco model H at a current of 5 mA. The protein concentration was 1.5 mg/ml, in a solution of 0.01 *M* Tris + 0.10 *M* NaCl.

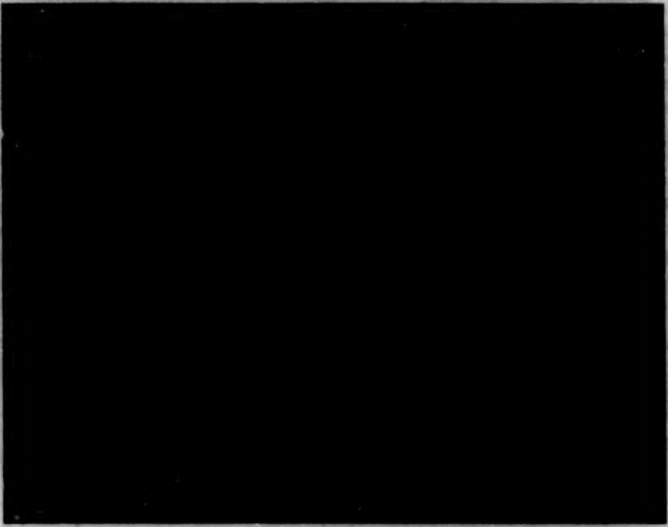

Fig. 8. Electron micrograph of purified alkaline phosphatase protein, magnified 163,000 times (taken by Prof. C. E. HALL[31]).

found localized in a single band, indicating the presence of only a single electrophoretic species of alkaline phosphatase. The isoelectric point again occurred at pH 4.5*.

Electron micrography: Fig. 8 shows the appearance of the purified enzyme preparation in the electron microscope. The particles appear to be of uniform size and approximately spherical in shape, and are not contaminated with a larger component. The diameter of the particles is 60 Å, in reasonable agreement with the value of 57 Å calculated for a spherical unhydrated protein particle of molecular weight 80,000. (The diameter was estimated from the length of the shadow—Prof. C. E. Hall, personal communication).

pH optimum: The effect of pH on the velocity of enzymic activity against NPP is shown in Fig. 9. The maximum velocity occurs at pH 8.0, and there is no detectable activity below pH 6.

Activation energy: The effect of temperature on the velocity of the enzymic activity as measured against NPP is shown in Fig. 10. The activity increases by a factor of 1.5 for a 10° increase in temperature. The activation energy for the reaction is 6880 cal./mole.

Heat inactivation: Crude cell extracts containing alkaline phosphatase can be maintained at 85° for at least 30 min without loss of enzymic activity, whereas the purified enzyme is unstable at the same temperature. It was found that the heat

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* In mammalian cells several electrophoretically different alkaline phosphatase molecules have been observed[30], whereas in *E. coli* we have detected only the single electrophoretic species after starch gel electrophoresis of crude cell extracts.

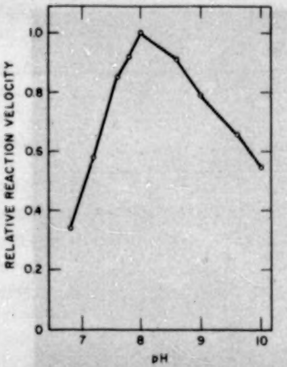

Fig. 9. Effect of pH on the rate of alkaline phosphatase activity against *p*-nitrophenyl phosphate The solutions were buffered with 1 *M* Tris and measurements were made at 25°. The enzyme concentration was 1 μg/ml.

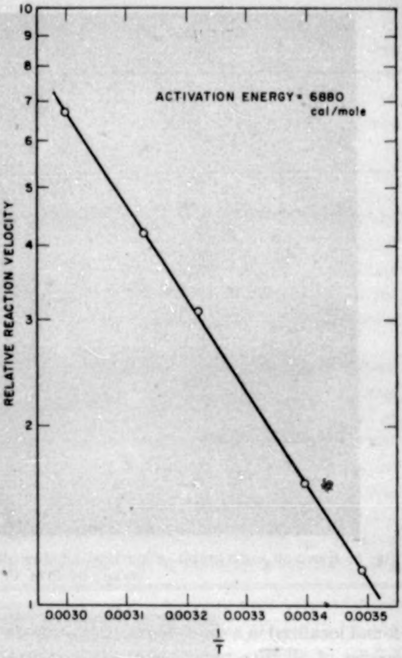

Fig. 10. Effect of temperature on the rate of alkaline phosphatase activity against *p*-nitrophenyl phosphate. The calculated activation energy is 6880 cal./mole. Measurements were made in 1.0 *M* Tris buffer at pH 8.0.

TABLE I

ACTIVITY OF ALKALINE PHOSPHATASE AGAINST DIFFERENT COMPOUNDS

The reaction mixtures contained 10^{-3} *M* substrate buffered at pH 8.0 with 1 *M* Tris (at this concentration the reaction velocity is independent of substrate concentration). Enzymic activity was measured by the release of orthophosphate.

Compound tested as substrate	*Relative reaction velocity*
p-nitrophenyl phosphate	1.0
Sodium β glycerophosphate	0.9
Glucose 1 phosphate	0.9
Adenosine 3′ phosphate	1.0
Adenosine 5′ phosphate	0.8
Cytidine phosphate	1.2
Guanosine phosphate	1.0
Uridine phosphate	1.3
Riboflavin 5′ phosphate	0.7
L-histidinol phosphate	0.9
Creatine phosphate	0
Adenosine triphosphate	0
Sodium pyrophosphate	0

stability for the purified enzyme could be raised to a level comparable to that of a crude extract by the addition of $MgCl_2$ or $MgSO_4$ to the medium. Fig. 11 shows the effect of Mg^{++} on the rate of inactivation of purified enzyme at 95°. With 0.01 *M* Mg^{++} in the medium, the rate of inactivation is about 15 times slower than that observed in the absence of Mg^{++}. Furthermore, in 0.01 *M* Mg^{++} medium the kinetics of inactivation is exponential over the range tested, which extended to 0.3 % residual activity. However, at lower concentrations of Mg^{++} the slope of the inactivation curve is not constant; initially the slope is greater than, and ultimately becomes equal to, the slope in 0.01 *M* Mg^{++} medium. This behavior indicates that in 0.01 *M* Mg^{++} medium the entire population of enzyme molecules is maximally stabilized to heat, while at lower Mg^{++} concentrations only a fraction of the population is stabilized.

Substrate specificity: The rate of phosphorolytic activity of the enzyme against several compounds, as measured by the release of orthophosphate, is shown in Table I.

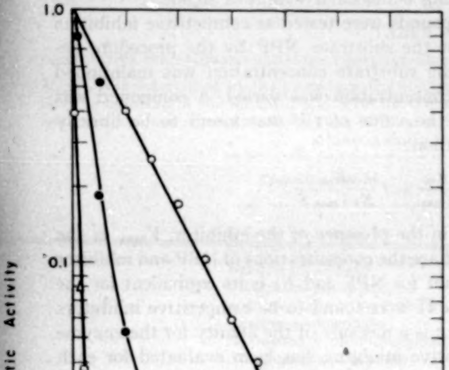

Fig. 11. Effect of Mg^{++} on the rate of thermal inactivation of alkaline phosphatase at 95°. The enzyme was equilibrated at 95° for the indicated times, after which the enzymic activity against *p*-nitrophenyl phosphate at 25° was measured. The solutions were buffered at pH 8.0 with 1 *M* Tris. The enzyme concentration was 0.1 mg/ml.

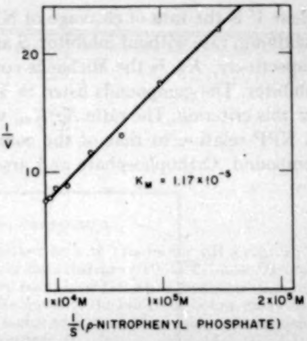

Fig. 12. Measurement of the Michaelis constant K_m of alkaline phosphatase with *p*-nitrophenyl phosphate. K_m was calculated from the equation:

$$\frac{1}{V} = \frac{1}{S}\frac{K_m}{V_{max}} + \frac{1}{V_{max}}$$

where *V* is the velocity of enzymic activity at a substrate concentration *S*, and V_{max} is the maximum velocity attainable with an excess of substrate. The experiments were carried out at 25° in 1 *M* Tris buffer at pH 8.0. The enzyme concentration was 0.2 μg/ml.

Activity was observed against all the phosphomonoesters tested, but the enzyme did not liberate orthophosphate from ATP, sodium pyrophosphate, or creatine phosphate. The organic moieties of these phosphomonoester substrates have only a small effect on the rate of enzymic activity. Alkaline phosphatase from cow's milk and calf intestinal mucosa have also been found to act as general phosphomonoesterases[27]. However, in contrast to the bacterial enzyme, these phosphatases were reported to be active against creatine phosphate.

Michaelis constant: A determination of the Michaelis constant with the substrate NPP is shown in Fig. 12. The value obtained at a pH of 8.0 and a temperature of 25° is $1.2 \cdot 10^{-5}$ (moles of NPP/l).

Turnover number: The turnover number calculated for a molecular weight of 80,000 is 2700 molecules of NPP cleaved/enzyme molecule/min at 25°. This value is lower, by a factor of about twenty, than the turnover number calculated for mammalian alkaline phosphatases[24, 27] assuming a molecular weight of 80,000.

Competitive inhibitors: Several compounds were tested as competitive inhibitors of alkaline phosphatase activity against the substrate NPP by the procedure described in Fig. 13. In this procedure the substrate concentration was maintained constant at $10^{-3}\,M$ and the inhibitor concentration was varied. A compound was classified as a competitive inhibitor if the value of $1/V$ was found to be linearly proportional to I, according to the equation:

$$\frac{1}{V} = \frac{1}{V_{\max}} + \frac{K_m}{V_{\max}} + I\,\frac{K_m}{K_I\,V_{\max}\,S}$$

where V is the rate of cleavage of NPP in the presence of the inhibitor, $V_{\max}$ is the maximum rate without inhibitor, S and I are the concentrations of NPP and inhibitor respectively, K_m is the Michaelis constant for NPP and K_I is its equivalent for the inhibitor. The compounds listed in Table II were found to be competitive inhibitors by this criterion. The ratio K_I/K_m, which is a measure of the affinity for the enzyme of NPP relative to that of the competitive inhibitor, has been evaluated for each compound. Orthophosphate and arsenate have the highest affinity for the enzyme,

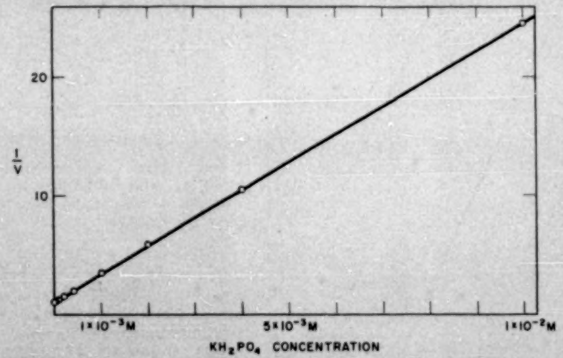

Fig. 13. Competitive inhibition by orthophosphate of the activity of alkaline phosphatase against *p*-nitrophenyl phosphate. The concentration of *p*-nitrophenyl phosphate was kept constant at $1 \cdot 10^{-3}\,M$. Measurements were made at 25° in $1\,M$ Tris buffer at pH 8.0.

three times greater than the affinity of NPP. Six other compounds tested, five of which are substrates for the enzyme (*cf.* Table I), have a lower affinity than NPP.

Evidence for a metal at the active site: Four metal chelating agents, EDTA (ethylenediaminetetraacetic acid), cyanide, 8-hydroxyquinoline, and 1,10-*o*-phenanthroline, were found to inhibit enzymic activity, as shown in Table III. The measurements reported in Table III were carried out immediately after diluting the inhibitor one hundred-fold, and under this condition EDTA was the only inhibitor whose effect was largely irreversible. However, the inhibition by EDTA could be reversed by exhaustive dialysis against Tris buffer.

It has been reported that an alkaline phosphatase from swine kidney is inhibited by EDTA and cyanide, and that this enzyme contains one atom of zinc per molecular weight of 37,000[24]. Assuming that the *coli* enzyme also contains a metal atom, and that the chelating agents act by binding to the metal, the following experiment suggests that the metal is located at the active site of the enzyme. If orthophosphate

TABLE II

COMPETITIVE INHIBITORS OF ALKALINE PHOSPHATASE ACTIVITY AGAINST *p*-NITROPHENYL PHOSPHATE

K_I/K_m was determined by the procedure described in Fig. 13. The value of K_I/K_m indicates the relative affinities of the inhibitor and NPP for the enzyme; the inhibitors which yield values of less than 1.0 have a greater affinity than NPP, and those which yield values in excess of 1.0 have a lower affinity than NPP.

Competitive inhibitor	*Inhibitor constant* K_I/K_m *at 25°*
Sodium phosphate	0.32
Sodium arsenate	0.32
Uridine phosphate	3.7
Guanosine phosphate	3.8
Sodium β glycerophosphate	4.2
Glucose-1-phosphate	5.3
Adenosine 5′ monophosphate	7.8
Adenosine triphosphate	8.0

TABLE III

INHIBITION BY METAL-CHELATING COMPOUNDS

Inhibitor and enzyme (0.5 mg/ml) were equilibrated together in 1 M Tris buffer pH 8.0 at 23° for the period indicated. Enzymic activity was assayed using the substrate NPP at a concentration of 10^{-3} M. The assays in which an inhibitor was present contained the same concentration of inhibitor as was used for equilibrating with the enzyme. Removal of the inhibitor before assaying was achieved by diluting the equilibration mixture by a factor of 100, and the assay was carried out immediately after dilution.

Inhibitor	*Inhibitor concentration*	*Minutes of equilibration of inhibitor with enzyme*	*% of initial enzymic activity*	
			Inhibitor present	*Inhibitor removed*
EDTA	$2.5 \cdot 10^{-3}$ M	20*	20	42
Sodium cyanide	$1 \cdot 10^{-2}$ M	10	0.7	90
	$1 \cdot 10^{-3}$ M	10	90	
8-hydroxyquinoline	$1 \cdot 10^{-3}$ M	10	2	88
1,10 *o*-phenanthroline	$1 \cdot 10^{-3}$ M	10	74	100

* The extent of inhibition by EDTA was found to depend on the time of equilibration; with longer equilibration the inhibition eventually reaches completion.

is present when the enzyme is equilibrated with EDTA, the enzyme is protected against the inactivating effect of EDTA. For example, equilibration of the enzyme with 0.09 *M* EDTA for 3 min at 30° reduces enzymic activity, as measured immediately after diluting away the EDTA, to 10 % of the initial value, but if 0.0005 *M* orthophosphate is present in the equilibration mixture the activity is reduced only to 70 %. It has been shown (*cf.* Table II) that the orthophosphate is capable of competitively inhibiting enzymic activity against the substrate NPP, which is an indication of the affinity of orthophosphate for the active site of the enzyme. Therefore, the protective effect of orthophosphate against inhibition by EDTA probably results from competition between the two substances for the active site.

the protective effect of orthophosphate against inhibition of the enzyme by EDTA probably results from competition between orthophosphate and EDTA for the active site.

DISCUSSION

It has been shown for several bacterial enzymes that the rates of enzyme synthesis are subject to regulation either by specific "inducer" substances, such as galactosides for the enzyme β-galactosidase[28], or by specific "repressor" substances, such as orthophosphate for the enzyme alkaline phosphatase[17]. The maximal rate of enzyme synthesis is obtained when the inducer is added to the growth medium, or when the repressor is removed. There is evidence from recent work on β-galactosidase that an inducer acts by competing with an intracellular repressor[29]. Therefore, both inducible and repressible enzymes may be controlled by a similar basic mechanism involving enzyme-specific repressors which reduce the rate of enzyme synthesis.

The maximal rate at which a bacterial cell synthesizes one of its enzymes, relative to the total rate of protein synthesis, has been measured for two of the enzymes of *E. coli*: for β-galactosidase[29] and, as reported in the present paper, for alkaline phosphatase. The β-galactosidase rate was determined with an inducer added to the medium, and the alkaline phosphatase rate with the repressor orthophosphate removed from the medium. The result in both cases is that the single enzyme constitutes about 6 % of the mass of cellular protein. The similarity between the two cases suggests that an *E. coli* cell may have the potentiality to synthesize any one of its proteins at a differential rate of about 6 % of the total, although it is unlikely that more than a few proteins could be synthesized simultaneously at this rate.

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We are indebted to Mrs. Akiko Higa for her technical assistance.

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MITOCHONDRIAL SWELLING AND ELECTRON TRANSPORT

I. SWELLING SUPPORTED BY FERRICYANIDE

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SUMMARY

The swelling of rat-liver mitochondria in presence of phosphate is inhibited by cyanide but restored by further addition of ferricyanide. The effects on swelling of inhibitors of electron transport (amytal, antimycin A) and of compounds which prevent phosphorylation (2,4-dinitrophenol, oligomycin) have been studied in presence and absence of ferricyanide and cyanide. It is concluded that, under defined conditions, swelling is supported by electron transport, even through a restricted portion of the respiratory chain, by a mechanism not directly involving the coupled phosphorylation process.

INTRODUCTION

That the swelling of isolated rat-liver mitochondria in the presence of phosphate or thyroxine is dependent on respiration is indicated by the following considerations.

With freshly-prepared mitochondria a variety of inhibitors of electron transport suppress swelling; thus, under appropriate conditions, antimycin A[1-3], amytal[1,2], oligomycin[2,4] and cyanide[1,3,5], when present in concentrations known to inhibit many mitochondrial oxidations, also prevent swelling. In the case of mitochondria which have been allowed to age by storing at 0° for one day in 0.44 M sucrose ("aged" mitochondria) it is necessary to add an oxidizable substrate, e.g. D-β-hydroxybutyrate, before phosphate or thyroxine can exert their effects. The inhibition by malonate of the swelling supported by succinate lends further support to the hypothesis that respiration is a necessary accompaniment of mitochondrial swelling[1].

It appears therefore that the interruption of the electron-transport chain at various points by the addition of specific inhibitors can prevent swelling. These observations do not indicate, however, whether swelling can be supported by electron transport in a restricted portion of the respiratory chain. We have therefore studied mitochondrial swelling when cyanide was present to inhibit the cytochrome oxidase and ferricyanide was added to act as terminal electron acceptor, and also when ascorbate was added to act as a substrate for the cytochrome *c*–cytochrome oxidase system.

Some parallel observations on the rate of reduction of ferricyanide by mitochondria are included in another communication[6].

MATERIALS AND METHODS

Materials

Water distilled in glass was used and, except where indicated otherwise, AnalaR reagents. Glass apparatus was cleaned with H_2SO_4. Ethanol was redistilled in glass. Tris(hydroxymethyl)aminomethane (tris) (Sigma 121) was recrystallized from ethanol. To prepare the buffer, tris was brought to pH 7.5 with HCl which had been twice redistilled in glass. 2,4-Dinitrophenol was synthesized from 1-chloro-2,4-dinitrobenzene and recrystallized 6 times; m.p. 112.5–113.5° (uncorr.). A neutral solution was prepared with addition of the requisite $KHCO_3$ and removal of the CO_2 *in vacuo*. To prepare sodium DL-β-hydroxybutyrate, the ethyl ester (Eastman Kodak) was distilled and the fraction 83 —4° (20 mm Hg) collected; this was saponified at room temperature with 0.8 N NaOH and the salt recrystallized 3 times from ethanol. Succinic acid was neutralized with KOH, and KCN with redistilled HCl. Potassium phosphate pH 7.5 was made from KH_2PO_4 and KOH. Antimycin A was obtained from the Wisconsin Alumni Foundation and oligomycin (a mixture of the A, B and C forms) was kindly given by Dr. B. C. PRESSMAN. L-Ascorbic acid (B.P. quality) was obtained from Roche Products Ltd.

As recommended by FONNESU AND DAVIES[7], the sucrose was deionized. 1750 ml of M sucrose were passed through a column, 21 mm in diameter, of Amberlite Monobed Resin MB-1 (50 ml when dry) in about 1.5 h. The acidic component (IR 120) catalyses the hydrolysis of sucrose, but when the solution was passed through at the rate stated the breakdown, as measured by the colorimetric method of SOMOGYI[8], was only about 0.2 %.

Mitochondria

Mitochondria were prepared from livers of male hooded Norwegian rats (180–250 g body weight; fed *ad libitum*), 0.44 M sucrose[9] being used throughout. The preparation was carried out in a room at 4°; all containers were immersed in ice and water, and the centrifuge set at —1°. Rapidly-cooled liver was finely cut with scissors and homogenized by hand (9 strokes) in a Potter-Elvehjem homogenizer with Perspex pestle (0.4 mm clearance on the diameter). The 10% (v/v) homogenate was centrifuged for 10 min at 600 × g. Application of the principles of DE DUVE AND BERTHET[10,11] allowed the sedimentation of the mitochondria at the M.S.E. Major refrigerated centrifuge without recourse to the high-speed attachment. The automatic starting device routinely fitted to this machine gives reproducible acceleration and hence facilitates the application of the required total (*i.e.* integrated) g-min. 50-ml plastic tubes were used throughout, with not more than 25 ml of suspension in each, in swing-out metal buckets; R_{max} was 20.1 cm. The centrifuge was set to run at 3,600 rev/min. The mitochondrial fraction was sedimented by a total of 39,200 g-min (R_{min}, 15.7 cm; S_{min}, 19,600 S). The particles in each tube (2.5 gequiv.) were then washed by addition of sucrose to 8 ml (R_{min}, 18.6 cm; 34,700 g-min; S_{min}, 7,800 S), and then once more in the same volume (43,100 g-min; S_{min}, 6,300 S). After the initial sedimentation of the mitochondria the supernatant was decanted, but the washings were removed by a pipette with a bent tip. The final suspension contained 0.5 gequiv. of mitochondria/ml (approx. 2 mg N/ml). It was stored in a narrow covered cylinder which was surrounded by ice and water in a vacuum flask which was kept in a refrigerator.

Measurement of swelling

Changes in extinction at 520 mμ (1-cm light path), read in a Beckman model DU spectrophotometer, were taken as a measure of swelling. Water from a thermostat was circulated through Beckman "thermospacers" on either side of the cell compartment and kept the latter at 20°. Six experimental cells were used in each run, one set of three being put into a narrow metal box immersed in the thermostat when the other set was in the cell compartment.

Small volumes of fluid were added to the cells by means of an Agla micrometer syringe fitted with glass delivery tube. 0.05 ml of mitochondrial suspension was added to 2.95 ml of incubation medium at zero time. In every case the medium was buffered with 25 mM tris-chloride pH 7.5 and contained 0.3 M sucrose (final concentrations). DL-β-Hydroxybutyrate when added was in 2 mM concentration, other substrates in 1 mM, inorganic phosphate in 10 mM, and cyanide and potassium ferricyanide each in 1 mM.

RESULTS

Fresh mitochondria

The swelling of fresh mitochondria induced by phosphate was suppressed by the addition of cyanide and was restored by the further addition of ferricyanide (Fig. 1). The rate of swelling was then almost as great as with phosphate alone, but the extent was slightly reduced. In the absence of phosphate, ferricyanide, with or without cyanide, produced no swelling. Hence in the presence of cyanide, both ferricyanide and phosphate must be present together in order to produce an effect.

Fresh mitochondria have been shown to contain intrinsic substrates, *e.g.* citrate[12], lactate[13] and precursors of glutamate[14], and to have a significant endogenous respiration[15]. Furthermore, endogenous respiration is still observed when ferricyanide is the terminal acceptor[4]. We suggest that the swelling of fresh mitochondria is supported by the oxidation of intrinsic substrates when either oxygen or ferricyanide is the ultimate acceptor, and that the experiment shown in Fig. 1 provides evidence that swelling may be supported by electron transport in a restricted part of the respiratory chain.

When DL-β-hydroxybutyrate was present the findings were similar to those depicted in Fig. 1, except that where swelling occurred it was somewhat more extensive. This difference has also been observed in the absence of ferricyanide[2]. The rate of reduction of ferricyanide in the presence of β-hydroxybutyrate is not affected by cyanide[6]. The failure of cyanide to decrease the rate of swelling produced by phosphate in presence of ferricyanide may be interpreted in terms of this observation.

Since mitochondria are stabilized by ethylenediamine tetraacetate[16] and other chelating agents[17] the possibility exists that cyanide may suppress swelling by combining with an essential metal. The finding that swelling, inhibited by cyanide, may be restored by ferricyanide, renders this unlikely.

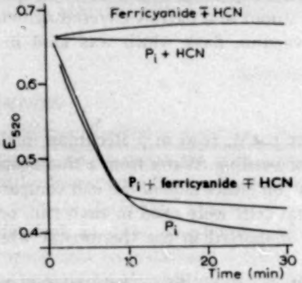

Fig. 1. The inhibition by cyanide of the swelling of freshly-prepared mitochondria in presence of phosphate (P_i), and the restoration of swelling by ferricyanide.

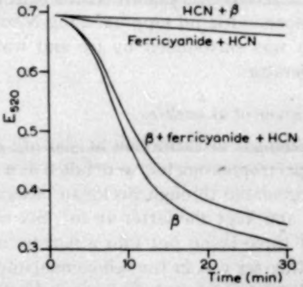

Fig. 2. The inhibitory effect of cyanide on the phosphate-induced swelling of aged mitochondria in the presence of DL-β-hydroxybutyrate (β). All cells contained phosphate. With cyanide present, swelling occurred only when phosphate, ferricyanide and substrate were all added as well.

Aged mitochondria

After the mitochondria had been stored at 0° in 0.44 M sucrose for 22–24 h, phosphate would not induce swelling in presence of cyanide and ferricyanide. On further addition of β-hydroxybutyrate rapid swelling occurred, almost equal in rate and extent to that obtained with phosphate and the substrate in the absence of cyanide and ferricyanide (Fig. 2). The pattern observed in the presence of substrate was therefore similar to that obtained with fresh mitochondria in absence of added substrate (Fig. 1). Succinate or L-glutamate also promoted swelling in presence of phosphate, cyanide and ferricyanide, but were much less effective than β-hydroxybutyrate (Fig. 3). In these experiments with aged mitochondria it has been shown that the process of swelling requires the simultaneous presence of a hydrogen donor (substrate) and an electron acceptor (ferricyanide) as well as a swelling agent (phosphate). The conclusion seems inescapable that swelling was supported by electron transport, in this instance along a restricted portion of the respiratory chain.

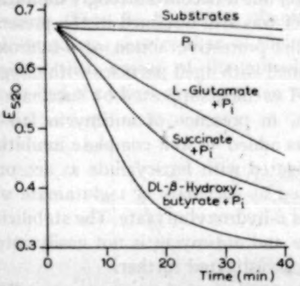

Fig. 3. The swelling of aged mitochondria in the presence of phosphate (P_i), ferricyanide, cyanide and the oxidizable substrates indicated. Swelling did not occur when phosphate or substrate was omitted.

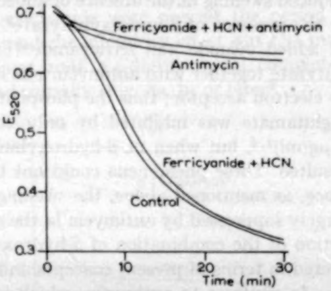

Fig. 4. The inhibition by antimycin A (20 mμg/ml) of phosphate-induced swelling of aged mitochondria in presence of β-hydroxybutyrate, with and without ferricyanide and cyanide. All cells contained phosphate.

Inhibition of swelling by antimycin A *and amytal*

With aged mitochondria the swelling induced by phosphate and supported by β-hydroxybutyrate was abolished by low concentrations of antimycin[1, 2]. This is seen in Fig. 4, which shows that antimycin (20 mμg/ml) had a similar effect when ferricyanide was added as terminal electron acceptor. Swelling supported by succinate and L-glutamate was also largely suppressed by antimycin under these conditions; indeed, the inhibition was more profound than was the case when oxygen served as terminal acceptor (Fig. 5). However, with the latter acceptor and in presence of succinate, the inhibition by antimycin becomes nearly complete when amytal is added

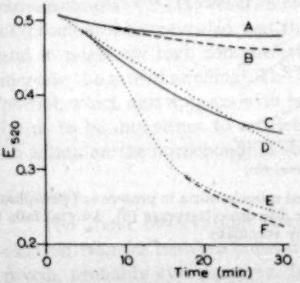

Fig. 5. The effects of amytal (2 m*M*) and antimycin A (20 mμg/ml) on the swelling of aged mitochondria in the presence of phosphate and succinate, with and without ferricyanide and cyanide. A, Antimycin, ferricyanide, cyanide; B, amytal, antimycin; C, ferricyanide, cyanide; D, antimycin; E, control; F, amytal.

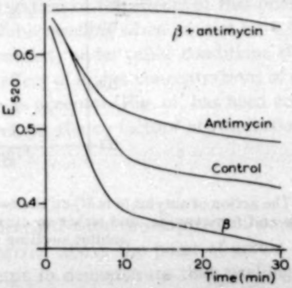

Fig. 6. The effect of antimycin A (20 mμg/ml) on phosphate-induced swelling of fresh mitochondria in presence of cyanide and ferricyanide, with and without β-hydroxybutyrate (β). Antimycin has little effect in the absence of β-hydroxybutyrate.

as well, despite the fact that amytal alone accelerates swelling (Fig. 5, 7). These findings are discussed below.

With fresh mitochondria antimycin has relatively little effect on phosphate-induced swelling in the absence of added substrate, but it becomes strongly inhibitory in the presence of β-hydroxybutyrate[2]. The effect was still obtained in the presence of added cyanide and ferricyanide (Fig. 6). This protective action of β-hydroxybutyrate together with antimycin was also obtained with aged particles with oxygen as electron acceptor; thus the phosphate-induced swelling supported by succinate or L-glutamate was inhibited by only 20 to 50% in presence of antimycin (10–50 mμg/ml)[1,2], but when DL-β-hydroxybutyrate was added as well complete inhibition resulted. These phenomena could not be investigated with ferricyanide as acceptor since, as mentioned above, the swelling supported by succinate or L-glutamate was largely suppressed by antimycin in the absence of β-hydroxybutyrate. The stabilizing action of the combination of β-hydroxybutyrate and antimycin is not easily interpreted in terms of present concepts and is being investigated further.

In contrast to antimycin, which inhibits the oxidation of succinate as well as of substrates with DPN-linked dehydrogenases by interrupting the respiratory chain in the vicinity of cytochrome *b*[18], the barbiturate amytal (5-ethyl-5-isoamylbarbiturate) suppresses the oxidation of the latter type of substrate while leaving the oxidation of succinate unaffected[19]. This compound is considered to act by inhibiting the electron-transport system between DPN and flavoprotein[18]. With ferricyanide as terminal electron acceptor the phosphate-induced swelling in the presence of β-hydroxybutyrate was completely suppressed by amytal, whereas that supported by succinate was even slightly accelerated (Fig. 7). These results parallel previous observations with oxygen as terminal acceptor[1,2].

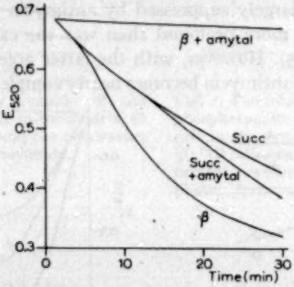

Fig. 7. The action of amytal (2 m*M*) on the swelling of aged mitochondria in presence of phosphate, cyanide and ferricyanide, and either succinate (succ) or β-hydroxybutyrate (β). Amytal fails to inhibit swelling supported by succinate.

Oligomycin and 2,4-dinitrophenol

The antibiotic oligomycin has been shown to inhibit the oxidation of β-hydroxybutyrate and glutamate in a mitochondrial system in which respiration was coupled to phosphorylation; the further addition of 2,4-dinitrophenol released the inhibition, indicating that oligomycin acts on the phosphorylation system itself[20]. Oligomycin

(0.4 μg/ml) had little or no effect on the phosphate-induced swelling of fresh mitochondria with oxygen[2,4] (Fig. 8a) or ferricyanide (Fig. 8b) as terminal electron acceptor. In the presence of β-hydroxybutyrate and still with fresh mitochondria oligomycin considerably reduced swelling, for some time, with both electron acceptor systems (Figs. 8a, b). When cyanide and ferricyanide were present the period of inhibition was shorter than in their absence, and the inhibitory effect was lost more readily with ageing of the mitochondria. 0.01 m*M* 2,4-dinitrophenol completely abolished the effect of oligomycin when added initially (Fig. 8a, b) or later[2].

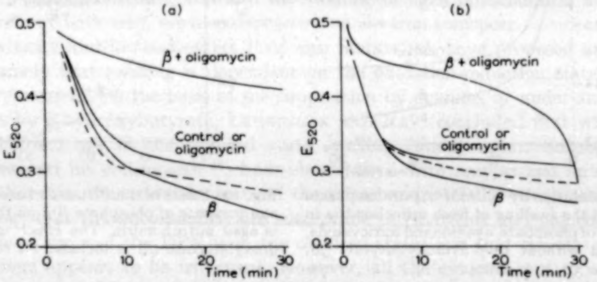

Fig. 8. The inhibition of the phosphate-induced swelling of freshly-prepared mitochondria by oligomycin (0.4 μg/ml) in the presence and absence of β-hydroxybutyrate (β), (a) without and (b) with cyanide and ferricyanide. In both (a) and (b), the broken curves show the response in presence of β-hydroxybutyrate and 0.01 m*M* 2,4-dinitrophenol, identical curves being obtained with and without oligomycin. Oligomycin inhibits only in presence of added substrate, and dinitrophenol releases this inhibition.

These results lead almost inevitably to the conclusion that it is the electron transport, and not its associated phosphorylation, which is a requirement for swelling under these various conditions. This might appear to be contradicted by the well-documented fact that dinitrophenol prevents mitochondrial swelling under some circumstances[2,3,16]. However, a closer investigation of the effects of this phenol and of dicoumarol has revealed that they only inhibit swelling when present both initially and in relatively high concentrations; in contrast, under other conditions they can increase the rate of swelling[4]. The inhibitory effect of higher concentrations of dinitrophenol, which also appears with ferricyanide as acceptor (Fig. 9), has been suggested by us to be due either to a deleterious effect on the co-factors of respiration or to an action on the mitochondrial structure itself[4].

Ascorbate

The above observations have been concerned with the support of swelling by electron transfer between substrate and a carrier above the point of action of antimycin, probably cytochrome *c*. In an attempt to demonstrate that swelling can be supported by electron transport between cytochrome *c* and oxygen, the effect of L-ascorbate on swelling of aged mitochondria in presence of phosphate was tested (Fig. 10). During 30 min either L-ascorbate alone (*cf*. ref. [21]) or phosphate alone had little effect but together they produced appreciable swelling. This was, however, much smaller than that due to phosphate together with β-hydroxybutyrate. If it can be assumed that these mitochondria can oxidize L-ascorbate in absence of added cytochrome *c*, and there is some evidence of this from LEHNINGER, HASSAN AND SUDDUTH, Table II[22], then Fig. 10 affords some evidence that swelling may be supported by electron transport beyond cytochrome *c*.

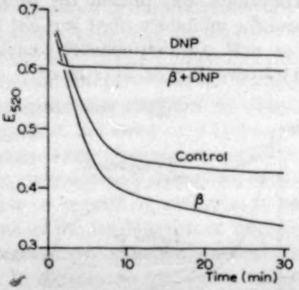

Fig. 9. Inhibition by 0.1 m*M* 2,4-dinitrophenol (DNP) of the swelling of fresh mitochondria in presence of phosphate, cyanide and ferricyanide, with and without DL-β-hydroxybutyrate (β).

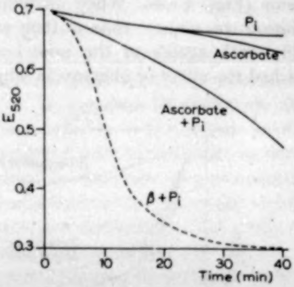

Fig. 10. Effect of 2 m*M* L-ascorbate in absence and presence of phosphate (P_i) on the swelling of aged mitochondria. The effect of DL-β-hydroxybutyrate (β) is included for comparison.

DISCUSSION

Dependence of swelling on electron transport

It seems clear that for swelling to occur in presence of the swelling agent phosphate the following conditions must be fulfilled: (a) presence of hydrogen donor, (b) presence of hydrogen acceptor and (c) electron transport through some part of the respiratory chain. The requirement for hydrogen donor is shown best by the fact that aged mitochondria will not swell unless an oxidizable substrate is added[1]. With fresh mitochondria we consider that the swelling is supported by endogenous respiration (p. 486), and strong evidence for this is provided by the inhibitory action of amytal and the restoration of swelling on further addition of succinate (Fig. 11a;

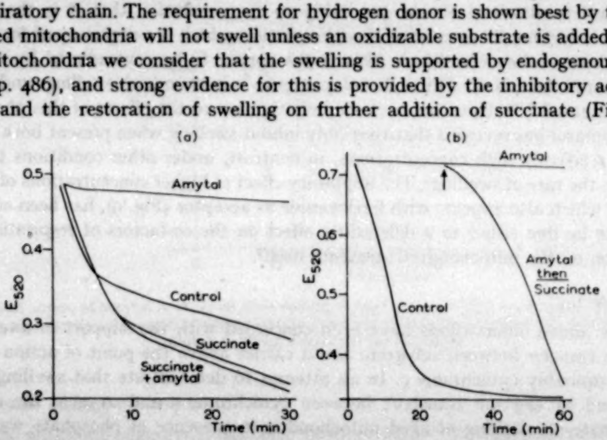

Fig. 11. (a) The phosphate-induced swelling of fresh mitochondria is inhibited by 2 m*M* amytal and restored by further addition of succinate. (b) The swelling of aged mitochondria in presence of phosphate and β-hydroxybutyrate is inhibited by 2 m*M* amytal. Addition of succinate after 25 min (arrow) initiates swelling.

see also Hunter, Fink and Hurwitz[23]). The requirement for hydrogen acceptor is shown by the experiments with ferricyanide in the present paper and by the abolition of swelling by the exclusion of oxygen[24]. The necessity for electron transport is established by the effects of inhibitors of the respiratory chain[1,2,5,23]. The restoration of swelling by ferricyanide after inhibition by cyanide and, possibly, the findings with ascorbic acid (p. 490) indicate that electron transport over even a restricted portion of the respiratory chain may be adequate. There is evidence, although somewhat less extensive than that for phosphate, that swelling induced by thyroxine[1,2,5,25] and phlorizin[26], and also the swelling of aged mitochondria in 0.2 M sucrose buffered with tris[1], are also dependent on electron transport.

Lehninger and his co-workers Ray and Schneider have proposed an alternative, namely that swelling is dependent on the oxidation-reduction state of the respiratory carriers. On the basis of the suppression by cyanide, or under anaerobic conditions by β-hydroxybutyrate, Lehninger and Ray[5] concluded that when the electron carriers are in the reduced state swelling cannot occur. Subsequently Lehninger and his colleagues[26,25], from observations with amytal and antimycin, deduced that it is the oxidation state of the mitochondrial DPN which is decisive for swelling; that the mitochondria are susceptible to thyroxine and phlorizin only when at least some of the DPN is in the oxidized state; and that the state of the other carriers appears to be irrelevant. However, all the evidence put forward by these workers is equally explicable by our thesis that the swelling is dependent on electron transport, but the suggestion that swelling is governed by the degree of oxidation of the bound DPN appears to be inconsistent with the following observations, which are explicable on the basis of the necessity for electron transport: (a) In the presence of phosphate or thyroxine, β-hydroxybutyrate or succinate enhance the swelling of fresh mitochondria and are necessary for that of aged[1]. The addition of an oxidizable substrate of this kind could scarcely be expected to lead to the oxidation of bound DPNH or, for that matter, of any other component of the respiratory chain. (b) With fresh mitochondria swelling when inhibited by amytal can be restored by succinate (Fig. 11a; see also ref. [23]), and this applies also to aged particles when the swelling is induced by phosphate together with β-hydroxybutyrate (Fig. 11b). Addition of succinate in presence of amytal could hardly lead to the oxidation of bound DPNH; on the contrary, addition of succinate has been shown to cause the reduction of bound DPN[27]. (c) The irrelevance of the extent of reduction of the DPN is further illustrated by an experiment briefly reported by Hunter, Fink and Hurwitz[23], who found that when swelling was inhibited by amytal it was not restored on further addition of α-oxoglutarate and ammonia although this resulted in 80 % oxidation of the DPNH.

Lehninger, Ray and Schneider[25] consider that information obtained from a study[21] of glutathione-induced swelling is at variance with our views. We have never maintained that swelling is dependent on respiration under all circumstances; we have, indeed, restricted our attention to swelling induced by phosphate or thyroxine and to the swelling of aged mitochondria which occurs in 0.2 M sucrose buffered with tris; nevertheless, the observations with glutathione are not necessarily inconsistent with our thesis. It is possible that glutathione acts as hydrogen donor as well as swelling agent. If the swelling depends on electron transport and if glutathione reacts directly with cytochrome *c*, the observed inhibition by cyanide and lack of inhibition by amytal and antimycin would be expected. However, it is necessary to know whether glutathione was indeed being oxidized in these experiments. Lehninger and Schneider[21], citing earlier work[22], state that oxidation of glutathione by mitochondria requires the addition of cytochrome *c*, but even a slow oxidation of glutathione in absence of cytochrome *c* might support the swelling. The rate of oxidation of β-hydroxybutyrate, for example, is also likely to be low with fresh mitochondria in absence of added phosphate acceptor and yet swelling is supported.

Oxidative phosphorylation and swelling

In our first communication[1], when we concluded that under various circumstances swelling is dependent on electron transport, we suggested, in view of the suppression by high concentrations of 2,4-dinitrophenol, that the effect of respiration may be mediated through the associated phosphorylation. As explained on p. 489, further experimentation has revealed that phosphorylation is unlikely to be involved in this way. Phosphorylation would seem to be involved only in so far as it controls the rate of respiration in a coupled system. This is illustrated by the inhibition of swelling by oligomycin as well as by some of the effects of dinitrophenol. It is noteworthy that after preincubation of the particles at 20° or longer storage at 0° the effect of oligomycin is no longer observed; this probably results from loss of the respiratory control. Furthermore, the inhibition by oligomycin is re-instated by the addition of magnesium ions[28] which are known to preserve the coupling of phosphorylation to respiration[29].

Swelling and hypothetical subsidiary electron-transport pathways

The overall pattern of the effects of inhibitors on swelling has been interpreted above in terms of the thesis that swelling is supported by electron transport along the accepted pathways. However, some of the effects of combinations of inhibitors, and of inhibitors in the presence of ferricyanide, cannot be fitted readily into the conventional scheme. The question therefore arises as to whether swelling may be supported by electron-transport pathways which differ from the established ones, either in the nature of the carriers themselves or in their availability to extra- or intra-mitochondrial substances. Pathways which differ from the accepted ones in the omission of one or more stages may be envisaged following Cooperstein's evidence[30] that soluble DPNH-cytochrome *c* reductase can reduce cytochrome oxidase directly. The rapid reduction of mitochondrial pyridine nucleotide by succinate[27] may also be cited. That pathways for the oxidation of different substrates should be wholly or partially segregated from each other, or differ in their availability to extra-mitochondrial solutes, is consistent with the finding[20] that certain antibiotics can inhibit the oxidation of one substrate more than that of another. Furthermore, it has been deduced that there are two mitochondrial pathways for the oxidation of DPNH which differ in their sensitivity to inhibitors[11,31,32].

We set out below some relevant observations on swelling and indicate how these could be explained in terms of a hypothetical subsidiary pathway or pathways. The effects of antimycin quoted are produced by concentrations (3–50 mμg/ml) which will inhibit swelling completely in presence of β-hydroxybutyrate.

1. *Succinate-O_2*: Partial inhibition of swelling by antimycin; almost complete inhibition by antimycin + amytal. Possible interpretation: existence of subsidiary

electron-transport path which is sensitive to amytal and insensitive to antimycin.

2. *Succinate-ferricyanide*: Almost complete inhibition of swelling by antimycin alone. Possible interpretation is conjunction with 1: the subsidiary path is sensitive to cyanide and not readily available to ferricyanide.

3. *Glutamate-O*$_2$: Complete inhibition of swelling by amytal, partial by antimycin. Possible interpretation: as under 1.

4. *Glutamate-ferricyanide*: Complete inhibition of swelling by amytal; almost complete inhibition by antimycin. Possible interpretation: as under 2.

A hypothetical scheme based on these considerations is shown in Fig. 12. Evidence for the existence of pathways of the type envisaged is being sought by measurements of oxygen uptake and ferricyanide reduction.

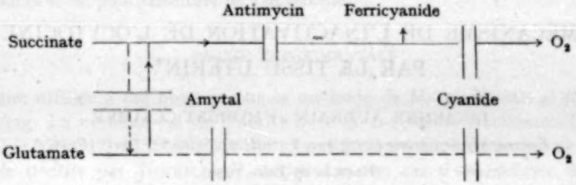

Fig. 12.

ACKNOWLEDGEMENT

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MÉCANISME DE L'INACTIVATION DE L'OCYTOCINE PAR LE TISSU UTÉRIN*

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(Reçu le 25 Juin, 1959)

SUMMARY

Mechanism of inactivation of ocytocin by uterine tissue

Ocytocin, when incubated under aerobic conditions with uterine tissue is never inactivated, whereas irreversible inactivation occurs rapidly when the incubation is performed under anaerobic conditions. The inactivation seems to proceed in two steps: the first step consists in a reduction of the disulfide bridge of ocytocin by uterine tissue; the second step is an irreversible inactivation of reduced ocytocin by an enzyme system, which is liberated into the medium during incubation. This inactivation proceeds either through proteolysis or through irreversible formation of mixed disulfides.

Similar experiments have been done with other organs, such as liver, kidney and diaphragm; it has been shown that ocytocin is inactivated by these tissues, but that inactivation in these cases proceeds equally with the reduced and the unreduced form of ocytocin. The physiological significance of these results is discussed.

INTRODUCTION

De nombreux auteurs ont étudié l'inactivation des hormones posthypophysaires par différents tissus. Les expériences ont porté soit sur l'ocytocine[1–5], soit sur la vasopressine[1,6]; une "ocytocinase" et une "vasopressinase", que les auteurs croient spécifiques** ont été décrites dans le plasma humain au cours de la gestation, par

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* Un exposé préliminaire de certains résultats du présent travail a été fait au IVe Congrès International de Biochimie (Vienne, 1958).

** La spécificité de l'ocytocinase du plasma humain a été récemment établie par TUPPY *et al.*[22,23] à l'aide de substrats synthétiques.

WERLE *et al.*[7,8]. Le mécanisme de cette inactivation pourrait consister, d'après DICKER ET GREENBAUM[6], soit en la rupture d'une ou de plusieurs liaisons peptidiques, soit en la réduction du pont disulfure de ces substances[9].

Dans un précédent travail[10], il a été établi que la réduction chimique du groupement disulfure de l'ocytocine provoquait une inactivation réversible de cette hormone. Alors que les travaux antérieurs avaient abouti à des conclusions contradictoires[12–16], RESSLER[11], dans une récente publication, confirme l'inactivation de l'ocytocine par réduction. Le présent travail a pour but d'étudier le mécanisme d'inactivation de l'ocytocine par le tissu utérin, sur lequel cette hormone exerce sa principale action physiologique; il montre que l'ocytocine, mise en présence de tissu utérin, subit une inactivation irréversible, mais que cette dernière, pour avoir lieu, doit être précédée d'une réduction du pont disulfure de l'ocytocine.

PARTIE EXPÉRIMENTALE

L'ocytocine utilisée a été obtenue par la méthode de MAIER-HÜSER *et al.*[17] et titre 125 U.I./mg. La cystéine est du chlorhydrate de L-cystéine (Hoffmann-La Roche). Toutes les opérations (réductions, incubations et dosages) ont été faites dans la solution de Tyrode décrite par JUNKMANN[18], additionnée en cas d'anaérobiose de 2 mg de glucose/ml[10]. Les incubations ont lieu à 37°, dans des fioles de Warburg, soit dans une atmosphère d'azote ou d'oxygène purs, soit dans l'air. Les réductions préalables de l'ocytocine par la cystéine sont faites dans les conditions décrites précédemment[10]. Les rats utilisés sont de souche Wistar et pèsent entre 130 et 160 g. Toutes les expériences du présent travail ont été faites avec des organes entiers, dans des conditions où l'ocytocine pouvait exercer son action sur le muscle au cours de l'incubation. L'ocytocine est dosée sur l'utérus de rat suivant la méthode de HOLTON[19]; l'activité résiduelle de l'ocytocine après réduction est dosée sur l'utérus de rat en anaérobiose[10], afin d'éviter la réoxydation de l'hormone. Une étude plus complète de cette méthode sera publiée ultérieurement.

RÉSULTATS

Inactivation de l'ocytocine par le tissu utérin en aérobiose et anaérobiose

La Fig. 1 montre l'inactivation, en fonction du temps d'incubation, d'une solution d'ocytocine incubée avec du tissu utérin de rat adulte. Les résultats indiquent que l'ocytocine est rapidement détruite en anaérobiose, alors qu'en présence d'oxygène, même après 3 h d'incubation, l'inactivation n'est pas significative[*].

Le Tableau I groupe l'ensemble des résultats obtenus en incubant l'ocytocine avec du tissu utérin dans l'oxygène, dans l'air ou dans l'azote. L'inactivation de l'ocytocine est toujours rapide en atmosphère d'azote et pratiquement nulle en atmosphère d'oxygène; les résultats sont inconstants avec l'air, ce qui peut s'expliquer par une anoxie partielle du tissu. Le fait que l'inactivation n'ait lieu qu'en anaérobiose pouvait

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[*] Note: des expériences préliminaires ont montré que, même en incubant pendant plusieurs heures en aérobiose, de l'ocytocine avec des quantités de tissu utérin telles que le rapport (poids du tissu/ocytocine) soit 50 fois plus élevé que celui indiqué sur la Fig. 1, aucune inactivation significative n'est observée. Ce résultat, obtenu avec des concentrations d'ocytocine proches de son seuil d'action physiologique ($10^{-10}-10^{-9}$ *M*) permet de supposer que l'ocytocine n'est pas inactivée lors de son action contracturante sur l'utérus en aérobiose.

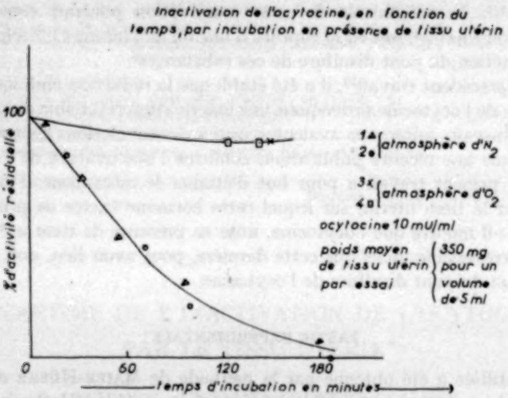

Fig. 1. Inactivation de l'ocytocine, en fonction du temps, en présence de tissue utérin; en abscisse: le temps exprimé en minutes; en ordonnée: activité résiduelle en %.

× ⊡ Echantillons incubés en atmosphère d'oxygène.

△ ⊙ Echantillons incubés en atmosphère d'azote.

Poids moyen du tissu: 350 mg par échantillon pour un volume de 5 ml.

TABLEAU I

INACTIVATION DE L'OCYTOCINE PAR INCUBATION AVEC DU TISSU UTÉRIN

Température d'incubation: 37°—milieu Tyrode pH 7—volume total 5 ml.

Concentration d'ocytocine dans le milieu réactionnel (mU/ml)	*Poids de tissu utérin dans chaque cellule (mg)*	*Temps d'incubation (h)*	*% d'activité résiduelle après incubation en atmosphère*		
			d'azote	*d'air*	*d'oxygène*
10	405	1	40	—	—
10	377	1	67	—	—
10	285	1	25	—	—
10	295	1	—	88	—
3.75	1,251	1.30	10	—	—
10	351	2	20	—	—
10	372	2	—	100	—
10	414	3	25	—	—
10	312	3	8	—	—
10	231	3	10	—	—
10	426	3	—	85	—
10	342	3	10	—	—
10	399	3	—	60	—
10	306	3	10	—	—
10	428	3	—	62	—
10	349	3	7	—	—
10	349	3	0	—	—
10	342	3	—	—	90
10	414	3	—	—	90

suggérer qu'il ne s'agissait que d'une simple réduction enzymatique de l'ocytocine; la réoxydation de l'ocytocine réduite devait alors provoquer la réactivation de l'hormone, comme dans le cas de l'inactivation de l'ocytocine par réduction de son pont disulfure au moyen de cystéine[10]: en fait il n'a jamais été possible de réactiver l'ocytocine inactivée par le tissu utérin. Il faut signaler d'autre part que l'incubation, avec ce même tissu, d'ocytocine réduite par la cystéine empêche sa réactivation ultérieure par oxydation.

L'ensemble de ces résultats montre qu'il doit y avoir intervention d'un mécanisme réducteur dans l'inactivation irréversible de l'ocytocine par l'utérus; on pouvait penser soit à l'activation, par réduction, d'un enzyme protéolytique à groupements -SH activables, tel qu'il en existe dans de nombreux tissus[20] et dont semblent se rapprocher l'"ocytocinase" et la "vasopressinase" déjà connues[3,7-9], soit à une réduction de l'ocytocine elle-même, qui serait suivie d'une protéolyse ou de la formation de disulfures mixtes inactifs du type de ceux décrits récemment par Ressler[11].

Inactivation de l'ocytocine réduite par un système enzymatique libéré par le tissu utérin

Le Tableau II montre qu'un système enzymatique inactivant l'ocytocine irréversiblement a pu être mis en évidence dans le milieu d'incubation des organes, après élimination de ces derniers, mais que seule l'ocytocine préalablement réduite par la cystéine est inactivée irréversiblement dans ces conditions. La nature enzymatique de la réaction est indiquée par la perte complète du pouvoir inactivant sous l'effet de la chaleur sur le milieu d'incubation (100°, 8 à 10 min).

TABLEAU II

INACTIVATION IRRÉVERSIBLE DE L'OCYTOCINE PAR LE MILIEU D'INCUBATION DES UTÉRUS

Les concentrations d'ocytocine sont experimées en milliunités/ml. La quantité d'enzyme libéré dans le milieu d'incubation provient de 80 mg de tissu utérin/ml pour les expériences 1, 3, 4, et à 40 mg/ml pour l'expérience 2.

Expérience No.	*Temps d'incubation minutes (37°) N_2*	*Ocytocine non réduite*				*Ocytocine préalablement réduite*					
		Initiale		*Finale*[*]		*Avant incubation*[**]		*Après incubation*[***]		*Témoin*[§]	
		Concn.	*%*	*Concn.*	*%*	*Concn.*	*%*	*Concn.*	*%*	*Concn.*	*%*
1	60	24	100	24	100	6	25	4	16.5	—	—
2	90	10	100	10	100	—	—	—	—	—	—
3	150	25	100	25	100	4.8	19	5.8	23	—	—
4	180	25	100	—	—	7.5	30	7.5	30	22.5	90

[*] Ocytocine non réduite incubée avec le système enzymatique du milieu d'incubation.

[**] Ocytocine réduite réversiblement par la cystéine; activité restante avant incubation; dosée sur l'utérus en anaérobiose.

[***] Ocytocine réduite identique à[**] incubée avec le système enzymatique et réoxydée avec O_2.

[§] Ocytocine réduite identique à[**] incubée avec le système enzymatique bouilli et réoxydée avec O_2.

Le Tableau III précise ces observations: dans cette série d'expériences, les utérus ont été d'abord incubés pendant 90 min à 37°, en anaérobiose stricte; le milieu d'incubation provenant des organes ainsi préincubés et contenant l'enzyme inactivant est alors réparti en plusieurs échantillons, de façon à étudier en parallèle l'action du

TABLEAU III

INACTIVATION IRRÉVERSIBLE DE L'OCYTOCINE PAR LE MILIEU D'INCUBATION DES UTÉRUS, EN PRÉSENCE DE CYSTÉINE

Les concentrations d'ocytocine sont données en milliunités/ml. La quantité d'enzyme libéré dans le milieu d'incubation provient de 100 mg de tissu utérin/ml pour les expériences 1, 2, 3 et 6, à 200 mg/ml pour l'expérience 4, et à 70 mg/ml pour l'expérience 5. Le milieu d'incubation est obtenu par préincubation des utérus en anaérobiose pendant 90 min. Les organes sont alors éliminés.

Expérience No.	*Temps d'incubation minutes (37°) N_2*	*Ocytocine non réduite*						*Ocytocine préalablement réduite*					
		Initiale		*Finale**		*Témoin***		*Avant incubation****		*Après incubation§*		*Témoin§§*	
		Concn.	*%*	*Concn.*	*%*	*Concn.*	*%*	*Concn.*	*%*	*Concn.*	*%*	*Concn.*	*%*
1	105	10	100	5	50	5	50	3.2	32	2.5	25	10	100
2	90	10	100	9	90	7	70	3.6	36	4.6	46	10	100
3	40	10	100	6.2	62	6.4	64	3	30	4.8	48	9.6	96
4	30	6.25	100	5 5	80 80	4	64	2.3	37	3.5 3.75	56 60	4.7	75
5	30	10	100	4	40	5.3	53	3	30	2 2.6	20 26	8	80
6	30	10	100	5.3 6	53 60	7	70	3	30	2 2.5	20 25	9	90
6	20	10	100	—	—	—	—	3	30	3.5	35	9	90
6	10	10	100	—	—	—	—	3	30	4.8	48	9	90

* Ocytocine non réduite incubée avec le système enzymatique du milieu d'incubation et une quantité de cystéine égale à celle de §.

** Ocytocine incubée comme en * sans système enzymatique, activité restante dosée sur l'utérus en anaérobiose.

*** Ocytocine réduite réversiblement par la cystéine; activité restante avant l'incubation dosée sur l'utérus en anaérobiose.

§ Ocytocine réduite identique à *** incubée avec le système enzymatique du milieu d'incubation et réoxydée avec O_2.

§§ Ocytocine réduite identique à *** incubée avec le système enzymatique du milieu d'incubation bouilli et réoxydée avec O_2.

système enzymatique inactivant sur l'ocytocine réduite ou non: (a) Le premier est incubé avec de l'ocytocine préalablement réduite par la cystéine; (b) Le second est incubé avec de l'ocytocine non réduite et une quantité de cystéine identique à celle apportée par l'ocytocine réduite du premier échantillon, afin de tenir compte d'un effet activateur éventuel de la cystéine sur le système enzymatique; (c) Le troisième est traité par ébullition et incubé ensuite avec de l'ocytocine réduite comme le premier échantillon; (d) Un témoin (sans milieu d'incubation), mesure la réduction de l'ocytocine par la cystéine au cours de l'incubation.

Tous ces échantillons sont d'abord incubés pendant des temps variables en anaérobiose stricte, afin de permettre l'inactivation enzymatique de l'ocytocine réduite ou non; l'activité de l'échantillon témoin est alors dosée en anaérobiose; les trois autres échantillons sont réoxydés pendant 120 à 180 min dans l'oxygène pur à 37°. L'activité ocytocique de ces échantillons est alors dosée.

Les résultats montrent que l'ocytocine réduite est inactivée irréversiblement par les enzymes contenus dans le milieu d'incubation, alors qu'avec ce même milieu bouilli la plus grande partie (80 à 100 %) de l'activité peut être retrouvée après réoxydation. L'ocytocine non réduite préalablement subit au cours de l'incubation une réduction de 30 à 50 %; l'inactivation irréversible de cette ocytocine par le tissu utérin est du même ordre de grandeur; cette inactivation est toujours très inférieure à celle de l'ocytocine préalablement réduite. Il apparaît donc que c'est bien l'ocytocine réduite qui sert de substrat à l'enzyme inactivant; il n'est pas possible de préciser si le système enzymatique en question possède des –SH activables par la cystéine.

Inactivation de l'ocytocine par différents organes du rat

Les "ocytocinases" et "vasopressinases" connues[7–9, 22, 23], si elles semblent parfois activables par des réactifs à groupes –SH, détruisent indifféremment l'ocytocine réduite ou non réduite; il en est de même de la plupart des tissus qui inactivent l'ocytocine[3, 9].

Le Tableau IV montre qu'en effet les coupes de foie et de rein inactivent rapidement l'ocytocine en aérobiose comme en anaérobiose. Les muscles striés comme le diaphragme n'inactivent l'ocytocine qu'en anaérobiose. Le mécanisme de cette inactivation n'est cependant pas identique à celui qui a été mis en évidence pour

TABLEAU IV

INACTIVATION DE L'OCYTOCINE PAR INCUBATION AVEC DIFFÉRENTS ORGANES

La concentration d'ocytocine dans les cellules est de 20 milliunités/ml. Les temps d'incubation ont été de 120 minutes dans tous les cas.

Nature de l'organe	*Poids de tissu dans chaque cellule (mg)*	*Atmosphère d'incubation*	*% d'activité résiduelle après 2 h d'incubation*
Foie	377	N_2	0
	330	O_2	1
Rein	151	N_2	10
	170	O_2	0
Diaphragme	334	N_2	15
	380	O_2	86
Utérus	237	N_2	23
	276	O_2	100

TABLEAU V

INACTIVATION IRRÉVERSIBLE DE L'OCYTOCINE PAR LE MILIEU D'INCUBATION DES DIAPHRAGMES EN PRÉSENCE DE CYSTÉINE

Temps d'incubation minutes (37°) N_2	*Ocytocine non réduite*					*Ocytocine préalablement réduite*					
	Initiale	*Finale**		*Témoin***		*Avant incubation****		*Après incubation§*		*Témoin§§*	
		Concn.	*%*	*Concn.*	*%*	*Concn.*	*%*	*Concn.*	*%*	*Concn.*	*%*
10	5	3.5	70	—	—	1.15	23	3.3	66	5	100
20	5	2.8	56	—	—	1.15	23	2.8	56	5	100
30	5	3.1	62	4	50	1.15	23	3.1	62	5	100
10	8	3.2	40	—	—	1.6	20	2.6	32	8	100
20	8	2.8	35	—	—	1.6	20	2.25	28	8	100
30	8	2.4	30	5	62	1.6	20	2	25	8	100

*, **, ***, §, §§ voir Tableau III.

l'utérus. En effet des expériences parallèles à celles qui ont été faites avec l'utérus (Tableau III) ont doné des résultats groupés dans le Tableau V.

Ces résultats montrent que le système enzymatique libéré par le diaphragme n'agit qu'en anaérobiose, mais attaque indifféremment l'ocytocine réduite et l'ocytocine non réduite.

DISCUSSION

L'ensemble de ces résultats montre que l'inactivation de l'ocytocine par le tissu utérin semble s'effectuer en deux étapes: en effet, une inactivation de l'ocytocine non réduite exige la présence de l'organe lui-même dans le milieu d'incubation et des conditions anaérobies, tandis que l'inactivation irréversible de l'ocytocine réduite par la cystéine peut avoir lieu sous l'influence du milieu dans lequel les organes ont été préincubés. La perte du pouvoir inactivant du milieu sous l'action de la chaleur indique qu'il s'agit d'une réaction enzymatique: la première étape serait donc une réduction du pont disulfure de l'ocytocine, alors que la seconde étape pourrait s'envisager, soit sous la forme d'une protéolyse, soit sous la forme d'une catalyse enzymatique de la formation de disulfures mixtes. Il semble cependant que la nature de la réaction et notamment sa totale irréversibilité plaident en faveur d'une réaction protéolytique; en effet, RESSLER[11] a montré que les disulfures mixtes d'ocytocine, obtenus par voie chimique, pouvaient être au moins partiellement réactivés par rupture de ces disulfures au moyen de réducteurs convenables et réoxydation de l'ocytocine libérée. Dans le présent travail des essais de réactivation de l'ocytocine ont été faits au moyen de cystéine et de 2-3-dimercaptopropanol (BAL), et ont toujours donné des résultats négatifs, ce qui rend improbable l'existence de disulfures mixtes d'ocytocine dans le milieu d'incubation. De toute façon, et quel que soit son mécanisme d'action, ce système enzymatique est inactif sur l'ocytocine non réduite.

Ce fait suggère qu'en présence de l'organe entier, l'ocytocine subit une réduction de son pont disulfure, qui la sensibilise à l'action ultérieure du système protéolytique. L'absence d'inactivation de l'ocytocine en aérobiose prouverait alors, soit que le système protéolytique n'est pas actif en milieu oxygéné et qu'ainsi l'ocytocine inactivée par réduction peut ensuite se réactiver par réoxydation, soit que l'ocytocine n'est pas réduite en aérobiose. Il est également possible que les deux mécanismes enzymatiques agissent en aérobiose, mais que la réoxydation de l'ocytocine par l'oxygène moléculaire soit suffisamment rapide pour empêcher sa destruction. L'étude séparée de ces deux phénomènes, à l'aide par exemple d'inhibiteurs spécifiques est compliquée par la nature du dosage sur l'organe isolé.

Il paraît difficile, dans l'état actuel de ce travail, d'établir sa signification physiologique. La protéolyse qui se produit sur l'ocytocine réduite inactive, et en absence du muscle utérin, ne peut évidemment avoir aucun rapport avec l'action hormonale de l'ocytocine sur l'utérus. Quant à la réduction de l'ocytocine qui exige la présence de l'organe intact, il est possible d'envisager qu'elle soit liée à la contraction de l'utérus sous l'action de cette hormone: en effet, l'intégrité du pont disulfure est nécessaire à l'activité hormonale de l'ocytocine[10] qui n'est pas inactivée par l'utérus en aérobiose au cours de son action; d'autre part, comme l'ocytocine réduite est autoxydable[21] et qu'ainsi sa réduction par le myomètre en aérobiose pourrait passer inaperçue, un mécanisme d'action catalytique de l'ocytocine sur un substrat spécifique du muscle utérin (par exemple une protéine à –SH de la membrane cellulaire) ne peut pas être exclu. Cependant, le présent travail ne permet d'apporter aucune preuve d'un tel mécanisme[*].

RÉSUMÉ

L'ocytocine, incubée avec du tissu utérin de rat pubère en aérobiose ne disparaît pas, tandis qu'en anaérobiose elle est inactivée rapidement et irréversiblement. Cette inactivation comprend vraisemblablement deux étapes: la première est une réduction du pont disulfure de l'ocytocine par le tissu utérin lui-même; la deuxième est une inactivation irréversible de l'ocytocine réduite par un système enzymatique libéré dans le milieu d'incubation; cette inactivation est due soit à une protéolyse, soit à la formation irréversible de disulfures mixtes.

Des essais semblables avec d'autres organes (foie, rein, diaphragme) ont montré que l'ocytocine était également inactivée, mais que cette inactivation se produisait indifféremment avec de l'ocytocine réduite ou non réduite. La signification physiologique de ces résultats est discutée.

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[*] Fong *et al.*[24] après l'étude de l'action de la vasopressine tritiée sur le tissu rénal, concluent également à une réaction d'échange entre le pont disulfure de la vasopressine et des groupements –SH d'une protéine réceptrice de la cellule.

ON THE [^{35}S]SULPHATE INCORPORATION IN FIBRINOPEPTIDE B FROM RABBIT FIBRINOGEN*

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SUMMARY

Two peptides, tentatively named A and B, are released during the coagulation of rabbit fibrinogen. Inorganic sulphate labelled with ^{35}S has been shown to be utilized *in vivo* by the rabbit in the synthesis of tyrosine-O-sulphate in one of the fibrinopeptides from fibrinogen. The half-life of this group in fibrinogen is found to be 70 h.

N-terminal analysis of rabbit fibrinogen and fibrin as well as of the fibrinopeptides has been performed, and the results compared with those found in other species.

INTRODUCTION

Fibrinogen is transformed into fibrin by the action of thrombin and other enzymes. During this process, limited proteolysis of the fibrinogen molecule occurs and different peptides are liberated[1-10]. At least two peptides, tentatively named A and B, are released from bovine fibrinogen. The release of peptide A occurs at a higher initial rate than that of peptide B. Furthermore, it has been shown that the release of peptide A runs parallel with fibrin formation, whereas peptide B is released at a maximum rate when fibrin formation is near completion[6-7]. This indicates that if proteolysis is necessary for the subsequent polymerization, splitting off of peptide A alone is indispensable for initiating polymerization of bovine fibrinogen. It was, in fact, found that a coagulating enzyme, reptilase, from the venom of *Bothrops jararaca* could quantitatively transform fibrinogen into fibrin, but nevertheless only split off peptide A[8,11].

Bovine peptides A and B show a definite difference in amino acid composition as well as structure[6,8,9,10a,10b,12].

In 1954, BETTELHEIM[13] found that peptide B contained tyrosine-O-sulphuric acid, this being, in fact, the first occasion on which the compound in question was identified in a protein. This finding has since been confirmed[7,14]. Tyrosine-O-sulphuric acid is known to be a normal constituent of urine[15]. By the use of ^{35}S-labelled sulphate and electrophoretic methods, it has been shown that sulphur is incorporated into the plasma proteins of the rat, especially α_1-globulins and albumins[16-18]. Since ^{35}S was also associated with the fibrinogen band during electrophoresis, incorporation into this protein as well seemed to occur. It was suggested that, in this case, the ^{35}S incorporation was due mainly to an incorporation into the sulphate group of tyrosine-O-sulphate[18].

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* Paper No. VI in a series of "Studies on ester sulphates".

In the present study, of which a preliminary report was given elsewhere[19], additional work has been done on the basis of the aforementioned observations. Thus, ^{35}S-labelled fibrinogen and a labelled fibrinopeptide containing tyrosine-O-sulphate were isolated from the plasma of rabbits given injections of ^{35}S-labelled sulphate. The half-life of the sulphate group in the fibrinopeptide was also estimated. Moreover, this isotope study provided some new data on the fibrinopeptides of rabbit plasma.

MATERIAL AND METHODS

Preparation of fibrinogen and fibrinopeptides

Rabbits weighing between 2 and 3 kg were used as experimental animals. In the *in vivo* isotope experiments, 1 mC of carrier-free ^{35}S-labelled sulphate per kg body weight was injected intravenously into one of the lateral ear veins.

The blood was withdrawn from one of the carotid arteries through a cannula inserted into the vessel, after anaesthetizing the animals with ether. As anticoagulant 4 % trisodium citrate ($\cdot 2H_2O$) was used (1 vol. of citrate solution + 7.3 vol. of blood). Usually, 100 ml of citrated blood were obtained from each animal.

In several experiments, radioactive sulphate was injected into groups of 4–10 rabbits, which were killed by exsanguination 24 h after the injection. The blood of all the rabbits was pooled, and fibrinogen and fibrinopeptides isolated as described in the following.

In the experiment carried out in order to estimate the half-life of the sulphate group in the fibrinopeptide, 5 groups of rabbits, 3 in each group, were sacrificed at varying intervals (4, 12, 24, 50 and 100 h) after injection of the isotope. The blood from the rabbits in each group was pooled, and the fibrinopeptides prepared as described below.

An isotope experiment *in vitro* was performed as follows. 200 ml of citrated blood was collected from untreated rabbits and mixed with 4.8 mC of ^{35}S-labelled sulphate. The mixture was incubated for 1 h at 37° in an atmosphere of an oxygen mixture (93.5 % oxygen + 6.5 % carbon dioxide). Fibrinogen and fibrinopeptides were also isolated from this blood.

Blood was also collected from untreated rabbits, for preparation of a non-labelled fibrinogen on which some of the chemical analyses were performed.

After collection, the blood was centrifuged at 3,500 rev./min in a Corda centrifuge for 20 min. When the plasma had been siphoned off, it was fractionated according to COHN's[20] method 6. The fibrinogen in fraction I was further purified according to the glycine method of BLOMBÄCK[21].

In the first experiments, the fibrinogen used (fraction I-0) had a coagulability of 82–90 %. In later experiments the fibrinogen was further purified (fraction I-2). This purified fibrinogen had a coagulability of 94–98 % as determined with a spectrophotometric method[22]. The same extinction coefficient ($E^{1\%}_{1\,\mathrm{cm}}$) at 282 mμ was used in the protein determinations as that found for bovine fibrinogen. In fraction I-0, the yield of fibrinogen from plasma was between 72 and 82 % (mean 75 %), the corresponding yield in fraction I-2 being between 59 and 75 % (mean 66 %).

For the preparation of fibrinopeptides from the fibrinogen, the wet fraction I-0 or I-2 was dissolved in 0.3 *M* ammonium acetate (pH 7) to a protein concentration of between 1.0 and 1.5 %. An equal volume of water was added, and immediately thereafter a bovine thrombin solution (100 NIH units/ml) to a final concentration of 1 NIH unit/ml. The coagulation mixture was left standing at room temperature (+ 20 to 22°) for approximately 6 h, whereupon the fibrin clot was collected on a Büchner funnel and thoroughly washed with water. The yield of fibrin under these conditions was usually in good agreement with that obtained when coagulation was performed under standard conditions (*cf.* ref. [22]). In the half-life study a lower yield was, however, obtained in all groups.

After acidification of the clot supernatant with formic acid to pH 3.0, it was put on a 2 % cross-linked Dowex-50 column (200–400 mesh), which had been previously equilibrated with 0.1 *M* ammonium formate–formic acid buffer*, pH 3.0. The inner diameter of the column was 0.9 cm. The length of the column varied with the amount of fibrinogen used for coagulation. For clot supernatants from 0.2, 0.5 and 1 g of fibrinogen, respectively, the following column lengths were used: 30, 50 and 70 cm. The column was eluted with ammonium formate–formic acid buffers* at pH values below 4, and with ammonium acetate–acetic acid buffers* above pH 4, as described earlier[7]. The elution rate was 1–6 ml/h. The pooled peaks were lyophilized, and the buffer salt removed by increasing the temperature to 40–50° during 5–7 h. The residue was dissolved in water and again lyophilized. A light, white powder was then obtained.

The combined biuret–phenol reagent of LOWRY *et al.*[23] was used for tracing the peptides eluted. Alternatively, the ninhydrin method of MOORE AND STEIN[24] was applied.

N-terminal amino acids

For N-terminal analysis of the fibrinopeptides, as well as of fibrinogen and fibrin, EDMAN's phenylthiocarbamyl method[25] was used. The details of the technique applied for fibrinogen and fibrin[26] and for fibrinopeptides[27] have been described previously.

Radioactivity determinations

The radioactivity of fibrinopeptide or fibrinogen solutions was determined by counting in a Geiger-Müller counter (2.3 mg/cm^2 mica end-window G.M. tube), after plating on frosted aluminium plates 0.25 ml of effluents from the ion-exchange chromatography, or (in the turnover study) of each fibrinopeptide preparation dissolved in 4 ml of distilled water.

Identification of tyrosine-O-sulphate

In order to establish the presence of tyrosine-O-sulphate in the rabbit fibrinopeptide B and its labelling *in vivo* by ^{35}S sulphate, the following procedures were undertaken.

1. *Ultraviolet absorption curves of the B peptide before and after acid hydrolysis*[7, 13]. Peptide B was dissolved in 0.1 *N* HCl at room temperature, and the absorption curve read in the region between 230 and 400 mμ, against 0.1 *N* HCl as blank. After increasing the HCl concentration to 1 *N*, samples were refluxed on the boiling water

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* 0.1 *M* with respect to ammonium.

bath for 4 and 75 min, and the ultraviolet absorption curve recorded after cooling the samples in running tap water. The peptide concentration was about 0.4 mg/ml.

2. *Chromatography.* 2 mg of rabbit peptide B and 20 mg of bovine peptide B, respectively, were hydrolyzed in baryta water for 24 h at 125°[13]. After neutralization with dilute sulphuric acid to pH 7, the barium sulphate precipitate was removed by high-speed centrifugation. Amino acids and the excess of barium ions were removed by passage over a Dowex-50 × 8 (200–400 mesh) column (1.2 × 1.2 cm) in the hydrogen cycle. After neutralization with ammonium hydroxide, the sample was lyophilized and subsequently used for the identification of tyrosine-O-sulphuric acid.

The alkaline hydrolysate was subjected to two-dimensional ascending paper chromatography on Whatman No. 1 filter paper in the frame described by DATTA, DENT AND HARRIS[28]. The following solvents were used: (a) Phenol–water (400 g + 100 g). A beaker containing concentrated ammonia was placed in the tank. (b) Butanol–2 *M* ammonia (250 ml + 250 ml).

After drying the papers in a stream of hot air from an electric hair-drying fan, the chromatograms of the labelled peptide were subjected to autoradiography on Gevaert Curix X-ray film and exposed for 1 week in a wooden screw-press. The X-ray films were then developed with Gevaert G. 150[29]. The papers were examined under the light of high-pressure mercury lamp. They were also developed with ninhydrin, and compared with chromatograms of an authentic specimen of synthetic tyrosine-O-sulphate and the alkaline hydrolysate of the fibrinopeptide B prepared from ox blood.

3. *Electrophoresis.* A similar comparative study was made by means of high-voltage paper electrophoresis (0.075 *M** sodium acetate–acetic acid buffer, pH 5.5, 30 V/cm, 1 h) of the alkaline hydrolysates (see 2.) combined with autoradiography.

RESULTS

Two rabbit fibrinopeptides were isolated by means of chromatography on 2 % cross-linked Dowex-50. A typical chromatogram of the clot supernatant of 932 mg of purified fibrinogen is shown in Fig. 1. The fibrinogen was prepared from rabbits which prior to the exsanguination had received ^{35}S-labelled sulphate. Both peptides, tentatively named A and B**, gave colour with the combined biuret-phenol reagent[23], or with the ninhydrin reagent[24]. In this chromatogram, peptide B was eluted from the column with 0.1 *M* formate buffer at pH 3.7, whereas peptide A was eluted with 0.1 *M* acetate buffer at pH 4.4. In other chromatograms the peptides were eluted at slightly different pH values, depending on the batch of resin used. In this experiment, 5.7 mg of peptide A and 4.7 mg of peptide B were obtained. In another experiment, the corresponding figures from 194 mg of fibrinogen were 1.6 and 1.8 mg. If the yield of 40–50 % as calculated below is considered, the peptides would constitute roughly 2–4 % of the fibrinogen.

Traces of glycine, used as reagent in the purification of fibrinogen, were found as a contaminant of the clot supernatant. The glycine was eluted from the column with 0.1 *M* ammonium formate buffers of pH 3.0 or slightly higher, and thus clearly separated from the B peptide.

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* 0.075 *M* with respect to sodium.

** The peptide containing tyrosine-O-sulphate has been denoted as peptide B, in view of its similarity in this respect to bovine peptide B. For the sake of simplicity the other peptide has been denoted as peptide A.

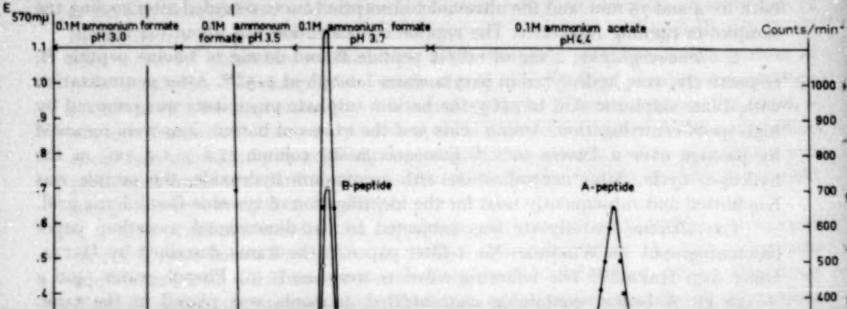

Fig. 1. Chromatogram of rabbit fibrinopeptides on 2 % cross-linked Dowex-50.

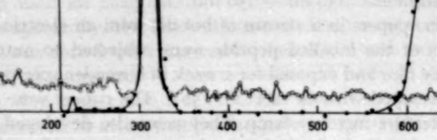

Fig. 2. Paper electropherogram of rabbit and ox peptides A and B. Electrophoresis was run in 0.1 *M* pyridine acetate buffer, pH 4.1, at + 8 to + 10° for 3 h, potential gradient about 20 V/cm. Whatman paper No. 3 MM was used. The spots were developed at 100° after spraying with a ninhydrin solution (0.2 % in acetone). The amounts of peptide applied to the paper were roughly 0.2–0.3 mg.

It is seen from Fig. 1 that the radioactivity was found exclusively in peptide B. No activity was present in peptide A. Variable amounts of inorganic sulphate were found in the solvent front of the chromatograms. In the *in vitro* isotope experiment, no incorporation of radioactivity occurred in fibrinogen or in fibrinopeptide B.

It can be inferred from the paper electropherograms shown in Fig. 2 that, at pH 4.1, peptide B had a stronger negative electric net charge than peptide A. Peptide B from rabbit fibrinogen differed markedly from the tyrosine-O-sulphate-containing bovine peptide B, in that it was much more acidic.

The ultraviolet absorption spectrum of rabbit fibrinopeptide B showed the same changes after hydrolysis as bovine peptide B. After acid hydrolysis, the typical tyrosine peak appeared, indicating that tyrosine occurs in a conjugated form in rabbit fibrinopeptide B (Fig. 3).

After alkaline hydrolysis of peptide B, ^{35}S-labelled tyrosine-O-sulphate could be demonstrated by means of two-dimensional paper chromatography or high-voltage electrophoresis, combined with autoradiography (Figs. 4 and 5). In both cases, the zone of radioactivity on the chromatogram corresponded exactly to the ninhydrin-positive spot and the fluorescent zone. It had the same localization as the ninhydrin-positive spot and the fluorescent zone of alkaline hydrolysates of bovine fibrinopeptide B, or of an authentic specimen of tyrosine-O-sulphate.

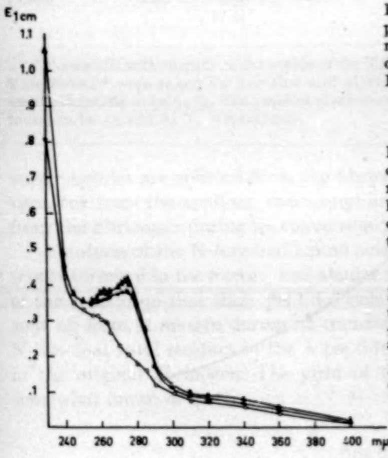

Fig. 3.

Fig. 3. Ultraviolet absorption properties of rabbit peptide B. ○—○, B-peptide in 0.1 *N* HCl at room temperature. ●—●, B-peptide after hydrolysis for 4 min in 1 *N* HCl at 100°. △—△, B-peptide after hydrolysis for 75 min in 1 *N* HCl at 100°.

Fig. 4. Chromato-autoradiogram on alkaline hydrolysate of ^{35}S-labelled fibrinopeptide B from rabbit. The zone delineated by a solid line is ninhydrin-positive and fluorescent, and is completely congruent with the localization of the radioactivity (black zone). For further details see text.

Fig. 5. Electrophero-autoradiogram on a synthetic specimen of tyrosine-O-sulphate (1 and 6), 20 and 40 μg, respectively, of alkaline hydrolyzed ^{35}S-labelled fibrinopeptide B from rabbit (2 and 3), ^{35}S-labelled inorganic sulphate (4) and 40 μg of alkaline hydrolyzed, unlabelled fibrinopeptide B from ox (5). The zones delineated by a solid line are ninhydrin-positive and fluorescent, and are (in 2 and 3) completely congruent with the localization of the radioactivity (black zone). For further details see text.

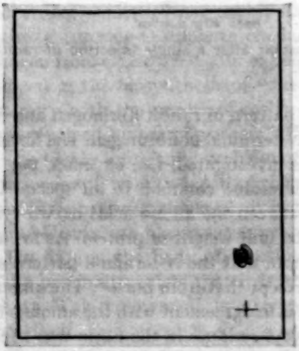

Fig. 4.

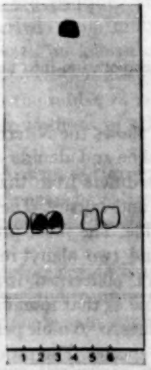

Fig. 5.

The results of the study of the half-life of the sulphate group of fibrinopeptide B are shown in Fig. 6, in which the specific radioactivity (counts/min/weight unit of peptide) is plotted against time after injection of the labelled sulphate. The maximum activity was recorded after 12–24 h, and was followed by a rapid decline. On the basis of the three last points of the decay curve, which approximately fits a 1st order reaction, the half-life of the sulphate group in the peptide was calculated to amount to 70 h. Curves of the same shape were obtained when the radioactivity was plotted against weight unit of fibrinogen or fibrin.

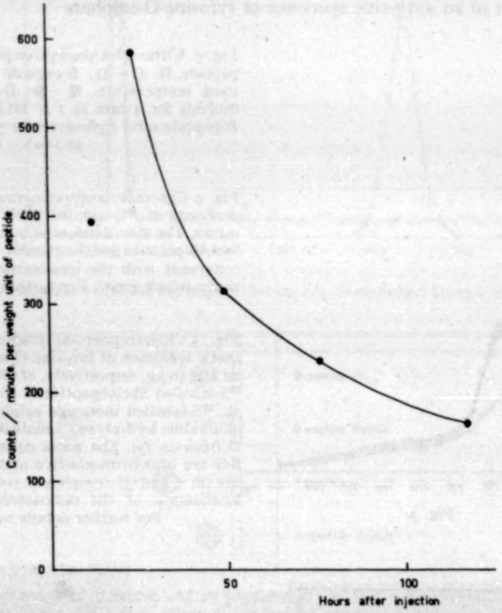

Fig. 6. ^{35}S incorporation into fibrinopeptide B in rabbit after a single injection of radioactive sulphate.

Table I shows the N-terminal amino acid pattern of rabbit fibrinogen and fibrin. Tyrosine, valine and alanine were found to be N-terminal in fibrinogen. The fibrinogen pattern thus differs from that of other species investigated, *i.e.*, ox, man, pig, goat, sheep, dog, and horse[26]. The only N-terminal residue common to all species is the tyrosyl residue. The most probable relation between the amino acids is two tyrosyl, two valyl and two alanyl residues in a 350,000 unit weight of protein. As far as the rabbit fibrin is concerned, it is interesting to note that the N-terminal pattern seems to be the same as that found in other fibrins, except that from horse[26]. The amount of tyrosyl residues, 0.56 mole per 10^5 g of protein, is in agreement with the amount found in the other species. The ratio of tyrosyl to glycyl residues in the fibrin was 1:2. The changes in the N-terminal pattern of fibrinogen and fibrin indicate that alanyl and

TABLE I

N-TERMINAL AMINO ACIDS IN RABBIT FIBRINOGEN AND FIBRIN

Figures in parentheses denote the relative amounts of different amino acids referred to tyrosine.

	Moles amino acid per 10^5 g protein							
Protein	*Tyrosine*		*Alanine*		*Valine*		*Glycine*	
	direct	*corr.**	*direct*	*corr.**	*direct*	*corr.**	*direct*	*corr.**
Fibrinogen	0.35	0.46 (1.0)	0.27	0.49 (1.06)	0.31	0.49 (1.06)	—	—
Fibrin	0.42	0.56 (1.0)	—	—	—	—	0.79	1.04 (1.9)

* Corrected with respect to the yields of the different amino acids. The figures of BLOMBÄCK AND YAMASHINA[26] were taken for tyrosine and glycine (77 and 76%, respectively). They found the yield of alanine to be 54%. The yield of alanine and valine determined in the same way by us was found to be 55 and 63%, respectively.

valyl peptides are split off from the fibrinogen during its conversion to fibrin. As is apparent from the analysis, two alanyl and two valyl peptides seem to be split off from the fibrinogen during its conversion to fibrin.

Analysis of the N-terminal amino acids in peptides A and B showed that valine was N-terminal in the former, and alanine in the latter. This affords definite evidence of the conclusion that these peptides isolated by chromatography are, in fact, those split off from fibrinogen during its transformation to fibrin. The over-all yield of the N-terminal valyl residues in the A peptide was calculated to be 50% of those found in the original fibrinogen. The yield of the alanyl residues in the B peptide was somewhat lower, or 40%.

DISCUSSION

In the present experiments, ^{35}S-labelled rabbit fibrinopeptide B was isolated, and ^{35}S-labelled tyrosine-O-sulphate was demonstrated in the peptide. These findings afford strong evidence that rabbit fibrinopeptide B derived from rabbit fibrinogen contains a tyrosine-O-sulphate residue, in agreement with its known occurrence in fibrinogen from cattle[8,13,14]. Our experiments have also shown that inorganic sulphate is utilized in the biosynthesis of tyrosine-O-sulphate in the rabbit *in vivo*. This is in conformity with the results of earlier studies on the biosynthesis of other ester sulphates reported by many workers[30–33].

The highly interesting problem of the site and mechanism of tyrosine-O-sulphate formation is still unsolved. Although many other ester sulphates have been synthesized in various *in vitro* systems of liver tissues, no report on the *in vitro* formation of tyrosine-O-sulphate has so far appeared[34].

The fate in the body of tyrosine-O-sulphate in fibrinopeptide B is not known, but the possibility exists that it is at least partially excreted in urine as tyrosine-O sulphate. This component is excreted in human urine in an amount of about 28 mg/day[15].

The question of the half-life of fibrinogen has been studied by several earlier workers, using different isotope methods[35-39]. In one type of experiment, labelled amino acids, *e.g.* ^{35}S-labelled methionine, in pure form or as yeast or yeast hydrolysates, have been used as tracer substances. In other experiments, plasma protein fractions, including purified fibrinogen labelled with ^{14}C, ^{35}S or ^{131}I, have been administered to experimental animals or human volunteers.

As recently discussed by GERDES AND MAURER[39], both these techniques are open to some criticism. In the experiments with labelled amino acids, some re-utilization of amino acids seems to occur. This was clearly demonstrated by MADDEN AND GOULD[35] in experiments with [^{35}S]methionine. When heavy doses of unlabelled methionine were given simultaneously, the re-utilization of [^{35}S]methionine was decreased. When labelled proteins are given, the re-utilization of labelled material is considered to be low. On the other hand, it is an open question whether or not exogenous serum proteins, if injected intravenously, can be expected to have the same turnover rate as the corresponding proteins found in the body[39].

Despite these methodological difficulties, fairly good agreement seems to be present between the figures for the turnover rate of fibrinogen reported by different workers (Table II). In all species studied, the half-life of fibrinogen was fairly short compared with that of other plasma proteins. If, as has been suggested, continuous coagulation and fibrinolysis take place within the blood stream, this rapid consumption of the fibrinogen is not surprising[40].

In the present study on the turnover rate of the ester sulphate group of the fibrinopeptide B, ^{35}S-labelled sodium sulphate was given as tracer substance. A similar technique has been used in studies on the turnover rate of the ester sulphate groups in sulphated mucopolysaccharides[32, 33, 41]. On the basis of these ^{35}S sulphate experiments

TABLE II

HALF-LIFE OF FIBRINOGEN AS ESTIMATED BY DIFFERENT WORKERS

Methods	*Genus*	*Estimated half-life (h)*	*Reference*
[^{35}S]methionine	Man	134	MADDEN AND GOULD, 1952[35]
[^{35}S]cystine	Man	161	VOLWILER *et al.*, 1955[37]
[^{35}S]methionine			
[^{35}S]yeast			
[^{35}S]yeast hydrolysate	Man	161	GERDES AND MAURER, 1957[39]
[^{35}S]methionine			
[^{35}S]yeast	Dog	101	MADDEN AND GOULD, 1952[35]
[^{14}C]proteins	Rabbit	63	DOVEY *et al.*, 1954[36]
[^{131}I]fibrinogen		66	
[^{14}C]fibrinogen	Rabbit	69	COHEN *et al.*, 1956[38]
[^{35}S]plasma		55	
[^{35}S]yeast			
[^{35}S]yeast hydrolysate	Rabbit		GERDES AND MAURER, 1957[39]
[^{35}S]methionine		86	
[^{35}S]sulphate	Rabbit	70	Present authors

in mammals, the extent of re-utilization of labelled material liberated in the course of the experiment must be considered to be very low. Firstly, it now seems to be fairly well established that the incorporation of labelled sulphur given as sulphate in other sulphur compounds than ester sulphates is extremely low, *cf.* ref. [33]. Secondly, a rapid decrease to low figures of labelled sulphate is known to occur in blood and tissues during the first 24–28 h after a single injection of inorganic sulphate[32,33]. Thirdly, the bulk of ^{35}S sulphate retained in the tissues probably occurs in the ester group of mucopolysaccharides[32,33]. Since the latter have been shown to have a comparatively long half-life[33,41], the leakage of inorganic sulphate from these sources is considered to be small.

In the present study, estimation of the half-life of fibrinopeptide B is based on the three last points of the decay curve in the time interval between 24 and 100 h after injection of radioactive sulphate. On the basis of earlier rat experiments[32,33], it is suggested that, at this time, the inorganic sulphate peak has been passed, and the labelling of inorganic sulphate in blood and tissues is too low to interfere significantly with the shape of the last part of the fibrinopeptide decay curve. This suggestion is also supported by the fact that the latter roughly fits a 1st order reaction curve.

For the aforementioned reasons, we conclude that the figure found in the present study for the half-life of the fibrinopeptide B of rabbit fibrinogen is close to the real figure. Good agreement can be noted between this figure and those for the half-life of the whole fibrinogen molecule of rabbit plasma reported by earlier workers (Table II). This seems to indicate that the part of the molecule belonging to peptide B is synthesized concurrently with the main part of the molecule.

During the transformation of fibrinogen from various animal species to fibrin, different species-specific peptides are split off. Thrombin seems to be an enzyme with a high specificity of action. It can be recalled that, out of the thousands of bonds in fibrinogen, only a few are split by thrombin. This enzyme has been found to hydrolyze tosylarginine methyl ester[42], and it has consequently been suggested to have a specificity for certain arginyl linkages in proteins. As far as bovine fibrinogen is concerned, both peptides A and B have arginine as C-terminal residue[9,10,12]. Since the initial rate of release of peptide A from fibrinogen is the same as the rate at which glycyl residues appear in the fibrin[8], it seems likely that thrombin splits arginyl–glycine linkages in this protein. Since the rabbit peptides A and B also contain arginine, it is fairly probable that an arginyl–glycine linkage is split here as well. Thus, thrombin would appear to have a trypsin-like specificity of action. However, when acting on fibrinogen the proteolysis brought about by thrombin is more limited than that by trypsin.

It has newly been demonstrated that only the splitting off of peptide A from bovine fibrinogen is indispensable for initiating polymerization. The release of peptide A runs parallel with fibrin formation, whereas peptide B is released at a maximum rate when fibrin formation is near completion[7,8]. Although the physiological importance of the release of bovine peptide B is not yet elucidated, recent experiments indicate that the release of fibrinopeptide B from bovine fibrinogen may induce a side-to-side aggregation of the fibrin units[43]. The rate at which the rabbit fibrinopeptides are split off is not yet known, but it would not be surprising if, here too, the A and B peptides were split off at different rates.

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STUDIES ON MUCOPROTEINS

I. THE STRUCTURE OF THE PROSTHETIC GROUP OF OVINE SUBMAXILLARY GLAND MUCOPROTEIN

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SUMMARY

Ovine submaxillary gland mucoprotein (OSM), the most potent of the influenza virus haemagglutinin inhibitors, was found to contain equimolecular amounts of N-acetylneuraminic acid and N-acetylgalactosamine comprising 42 % of the mucoprotein. The prosthetic group was isolated and shown to be the disaccharide 6-α-D-N-acetylneuraminyl-N-acetylgalactosamine by elementary analysis, identification of its components, susceptibility to α-neuraminidase and periodate consumption. The derivative 6-α-D-N-acetylneuraminyl-anhydro-N-acetyl-galactosamine, a chromogen in the direct Ehrlich reaction in the cold, formed from the prosthetic group on gentle alkali treatment, was also prepared. 93 % of the total galactosamine of OSM was oxidized by periodate, and 85 % of the N-acetylneuraminic acid was released by α-neuraminidase concomitant with the loss of biological activity.

INTRODUCTION

The mucoproteins produced by the submaxillary glands of various animal species have been the subject of many investigations (for historical review see GOTTSCHALK[1]). They are the richest and most convenient source of the various types of sialic acid and they were early recognised as potent inhibitors of haemagglutination by influenza viruses[1]. Whereas the sialic acid containing mucoproteins of the serum type, for instance orosomucoid, have a very complex oligo- or polysaccharide as prosthetic group[2], the submaxillary gland mucoproteins contain a relatively simple carbohydrate. For this reason the glandular-type mucoproteins lend themselves more to structural studies of their prosthetic group than do the serum mucoproteins. The main sugar components of the submaxillary gland mucoproteins are N-acetylgalactosamine (NAGal) and sialic acid[3-6]. Bovine submaxillary gland mucoprotein (BSM), as prepared in our laboratory, contains 22.4 % sialic acid (expressed as N-acetylneuraminic acid) and 12.7 % D-galactosamine, *i.e.* about equimolecular quantities of the two components; in addition small amounts of D-glucosamine (2.1 %), D-galactose (0.7 %), L-fucose (0.7 %) and D-mannose (trace) are present[7]. In a previous paper[8] it was shown (a) that the prosthetic group of BSM is a simple disaccharide consisting of NAGal and sialic acid, (b) that within the disaccharide sialic acid is linked through an α-ketosidic linkage to carbon atom six of NAGal and (c) that the disaccharide is split into its components by α-neuraminidase.

Though BSM inhibits in high dilution haemagglutination with the PR8 strain of influenza virus as indicator, it is practically without effect on the agglutination of red blood cells when tested with other strains of influenza virus as indicators[9]. Ovine submaxillary gland mucoprotein (OSM), on the other hand, is the most potent virus haemagglutinin inhibitor obtained so far[10], and is active against a whole spectrum of indicator viruses. Chemically OSM differs from BSM by the type of sialic acid incorporated in the prosthetic group. Whereas the sialic acid of BSM is a mixture of ON-diacetylneuraminic acid, N-acetyl-O-diacetylneuraminic acid and N-glycolylneuraminic acid, the sialic acid of OSM is nearly exclusively N-acetylneuraminic acid (NANA) with only a very small percentage of N-glycolylneuraminic acid[11]. Also it is easier to prepare from the sheep submaxillary glands a mucoprotein free or practically free from sugars other than NAGal and sialic acid than to isolate such a mucoprotein from the corresponding bovine glands. For these reasons, OSM, a typical glandular mucoprotein, was chosen for a more detailed investigation of its structural features. This paper is concerned with the composition and structure of the prosthetic group.

EXPERIMENTAL

Materials

The crystalline compounds N-acetyl-D-galactosamine, D-galactosamine hydrochloride, N-acetylneuraminic acid and pyrrole-2-carboxylic acid were the preparations used in the previous paper[8].

The other sugars were standard preparations exhibiting a single spot on paper-chromatographic examination.

Chromogens I and III were prepared from N-acetyl-D-galactosamine according to Kuhn and Krüger[12].

Ovine submaxillary gland mucoprotein (OSM). Fresh ovine submaxillary glands were freed from fat and connective tissue, cut into cherry-size pieces, washed on a Büchner funnel, covered with an equal weight of water and allowed to stand overnight at 0°. After removal of the liquid the cut glands were passed through a coarse meat mincer and extracted 3 times at 1-h intervals with an equal weight of water at 0°. These extracts were discarded to avoid contamination of the final product with blood. The resulting well-washed glandular material was then extracted 4 times with an equal weight of water at 0° for 48 h. These extracts were processed by the method of Curtain and Pye[9] shown by the authors to result in an electrophoretically homogeneous product. As described for the preparation of BSM[8], the addition of two further steps to the procedure was found to improve the product. When 1000 g of cleaned glands were submitted to this procedure, the following yields of lyophilized OSM were obtained: first extract 5 g, second extract 3 g, third extract 2 g and fourth extract 1.5 g.

Sialic acid from OSM was obtained as described for BSM[8].

Indicator viruses were prepared according to standard procedure[13].

Vibrio cholerae neuraminidase (RDE) was prepared in a highly purified state by Drs. ADA AND FRENCH and kindly presented by them; 7 μg protein were equivalent to 10.000 units.

Ion exchange resins were used in columns of appropriate size. Dowex 50-X4 (50–100 mesh) was prepared and regenerated according to BOAS[14]. Dowex 1-X2 (50–100 mesh) was converted to the formate form by the procedure of SVENNERHOLM[15].

METHODS

The non-amino sugars were determined by the method of GOTTSCHALK AND ADA[5].

Sialic acid and glycolic acid were determined as described previously[8]. All sialic acid values are expressed as N-acetylneuraminic acid (NANA).

Hexosamine determinations were carried out according to RONDLE AND MORGAN[16] on the vacuum-dried acid hydrolysate of the test material. Unless otherwise stated all hexosamine values refer to the free base. In order to liberate hexosamine from OSM, the latter was hydrolysed with 7.4 N HCl for 10 h at 100° in a sealed tube. When D-galactosamine hydrochloride was submitted to the same treatment, 88 % of the hexosamine was recovered. All hexosamine values were corrected accordingly.

Periodate oxidation of the disaccharide was carried out as described previously[8]. Periodate oxidation of OSM was performed at pH 5 with excess of potassium and sodium metaperiodate respectively at specified temperatures and times. The excess periodate was reduced by ethylene glycol and the mixture exhaustively dialysed against water.

Standard conditions for RDE treatment of substrates were pH 6.5, 0.005 M Ca^{++}, 35° and 20 h.

Dry matter: an aliquot of the mucoprotein solution was dried at 92° and weighed.

Paper partition chromatography was carried out according to GOTTSCHALK AND ADA[5]. The standard solvent used was *n*-butanol–pyridine–water (6:4:3, v/v). When a compound was chromatographed in more than one solvent, the second solvent was *sec.*-butanol–acetone–acetic acid–water (6:6:3:5, v/v).

Preparation of the prosthetic group of OSM

5 g of lyophilized OSM was dissolved in 500 ml water and the pH adjusted to 8 with saturated barium hydroxide solution. The mixture was quickly heated to 80° in a boiling water bath, 0.95 g $Ba(OH)_2 \cdot 8\ H_2O$ added and the mixture kept for 10 min in a water-bath at 80°. The solution was ice-cooled, acidified to pH 4 with N H_2SO_4 and dialysed at 0° against 10 volumes of water for 60 h with occasional stirring. The dialysate, adjusted to pH 4 with N H_2SO_4, was applied directly to a Dowex I-formate column (resin bed 20 × 200 mm). The column was washed with water until the positive direct Ehrlich reaction of the effluent, due to the presence of chromogen I, as established chromatographically, disappeared.

The compounds adsorbed to the resin were eluted with formic acid increasing in concentration from 0.1 N to 0.4 N. The eluate was collected in 12-ml fractions using an automatic fraction collector. The first 564 ml of eluate was orcinol-negative and did not give a direct Ehrlich reaction; it was discarded. The next 108 fractions were orcinol-positive. Each sixth fraction was concentrated *in vacuo* and chromatographed. From the colour reactions of the chromatographed material it was seen that fractions 1–18 consisted almost entirely of NANA and that fractions 54–108 contained mainly substances reacting both with direct Ehrlich reagent in the cold and with orcinol reagent.

Fractions 1–18 were pooled and lyophilized; yield 30 mg: pool I.

Fractions 19–53 were also pooled and lyophilized; yield 130 mg: pool II. When a sample of pool II was chromatographically analysed before and after treatment with α-neuraminidase (RDE), the main component was found to be NANA linked to NAGal; in addition small amounts of free NANA and of NANA bound to chromogens (in the direct Ehrlich reaction in the cold) were present.

Fractions 54–108 were combined and lyophilized; yield 110 mg: pool III.

Pool I was used for the purification and crystallization of NANA according to CORNFORTH, FIRTH AND GOTTSCHALK[17].

Pool II was used for the separation of N-acetylneuraminyl-NAGal (NANA-NAGal). This separation was effected by applying the material of the pool to Whatman 3 MM paper, 2 mg/in. paper, and developing with butanol–pyridine–water. From the thoroughly air-dried paper the area shown on a guiding strip to contain the disaccharide was cut out and the strip eluted with water at 0°. The eluate was applied to a small Dowex I (formate form) column, the column rinsed with water and elution carried out with 0.3 N formic acid at 0°. The orcinol-positive eluate was lyophilized and kept over P_2O_5 in vacuo at 0°.

Pool III was used for the isolation of N-acetylneuraminyl-chromogen I (NANA-chromogen I) and of N-acetylneuraminyl-chromogen III (NANA-chromogen III) by paper chromatography as above. The pool contained NANA-chromogen I, NANA-chromogen III and some NANA-NAGal. While NANA-chromogen I separated well on paper from NANA-chromogen III, it did not separate from NANA-NAGal sufficiently for satisfactory elution. For this reason, only fractions 82–108, practically devoid of NANA-NAGal, were applied to paper for isolation of NANA-chromogen I.

When the alkali treated OSM, after dialysis, was submitted to a second alkali treatment as above, the following yields were obtained: 20 mg (pool I), 100 mg (pool II) and 80 mg (pool III).

The combined dialysates resulting from a third and fourth alkali treatment of the mucoprotein material yielded about 240 mg substance which on chemical analysis was found to contain 35 mg hexosamine corresponding to 40 % NANA-NAGal of the total yield. This value compared well with the NANA-NAGal content of the material obtained from the first and the second alkali treatment. Paper chromatography of the material from the third and fourth alkali treatments before and after RDE action showed that it consisted of NANA, NANA-NAGal and NANA-chromogens I and III in approximately the same relative proportion as the material obtained from the first and second treatments.

RESULTS

Sugar analysis of OSM

The analytical values for the component sugars of OSM after appropriate hydrolysis are summarized in Table I.

The sialic acid of OSM is known to be NANA[18]. Determination of the glycolyl content of sialic acid isolated from OSM showed that 0.5 % of the total sialic acid was N-glycolylneuraminic acid. Chromatographic analysis of the isolated hexosamine of

TABLE I

COMPONENT SUGARS OF OSM

Values are expressed in percentage of dried OSM.

Sialic acid	*Hexosamine*	*Galactose*	*Mannose*	*Fucose*
25.4	15.0	0.30	0.15	0.40

OSM showed it to be galactosamine only; in some preparations a trace of glucosamine was seen. In all preparations ninhydrin oxidation of the isolated hexosamine revealed on chromatographic analysis of the oxidized material a single sugar spot coinciding in R_F value and colour shade with D-lyxose.

Identification of the prosthetic group

Elementary analysis and general properties: The material isolated from pool II was snow-white, had strong reducing power and, when dissolved in water, gave a very acid solution. It reacted with both the orcinol and indirect Ehrlich reagents and gave the direct Ehrlich reaction on heating.

For analysis (Microanalytical Laboratory, Australian National University) the material was dried in high vacuum over P_2O_5 at room temperature for several days; on further drying at 55° in high vacuum over P_2O_5 no loss in weight was observed.

	C	H	N
Found: preparation 1	44.51	6.29	5.68
Preparation 1 after heating at 55°	44.47	6.29	
Preparation 2	44.62	6.34	5.24
Calc. for $C_{19}H_{32}O_{14}N_2$ (512.47)	44.53	6.29	5.47

Identification of the components: 15 mg of the material, dissolved in 2.0 ml 0.1 *N* H_2SO_4, was heated at 80° for 60 min. After cooling the solution was applied to a Dowex 1 (formate form) column and the effluent collected and dried. The column was then eluted with 0.3 *N* formic acid and the eluate lyophilized.

N-acetylgalactosamine: When a sample of the dried effluent was chromatographed in butanol–pyridine–H_2O, the indirect Ehrlich spray showed an intense purple spot at R_F 0.36 coinciding in R_F value and colour shade with NAGal standard (the NAG standard had an R_F value of 0.41) and a weak purple spot at R_F 0.45 (most probably N-acetyltalosamine). On borate-treated paper and with the same solvent purple spots at R_F 0.09 (NAGal) and R_F 0.13 (most probably NATal) were observed (NAG standard R_F 0.19).

When 9.6 mg authentic N-acetyl-D-galactosamine was treated with $Ba(OH)_2$ solution, pH 11.1, at 80° for 10 min and the cations removed, chromatography resulted in spots, reacting with indirect Ehrlich spray, at R_F 0.36 (strong) and R_F 0.45 (weak) as above, the ratio of their colour intensities being 10:1. The R_F value of 0.45 is that given in the literature for N-acetyltalosamine[19].

During the gradual release of the prosthetic group from OSM by alkali an even smaller degree of conversion of NAGal to NATal occurred. Our best preparations contained only a few percent of the stereoisomer.

The residual effluent (see above) was heated in 2 *N* HCl at 100° for 5 h and the solution dried. After oxidation of the dried residue with ninhydrin chromatography revealed only a single reddish spot at R_F 0.42 (lyxose standard R_F 0.42, arabinose standard R_F 0.35), when sprayed with aniline hydrogen phthalate.

N-acetylneuraminic acid: The lyophilized eluate (about 7.5 mg) was dissolved in 4 ml 0.1 *N* NaOH and heated at 100° for 20 min. The solution, adjusted to pH 8, was twice extracted with ether and the extract discarded. The solution was then brought to pH 3 with dilute H_2SO_4 and extracted several times at this pH with ether. The combined ether extracts were washed with water and dried over anhydrous Na_2SO_4. The material extracted with ether was dissolved in ethanol; its absorption spectrum and that of authentic pyrrole-2-carboxylic acid are shown in Fig. 1. From the molecular extinction coefficient of pyrrole-2-carboxylic acid[20] it was calculated that the ether extract contained 0.146 mg pyrrole-2-carboxylic acid, *i.e.* 5.4 % of the NANA present was converted to the pyrrole.

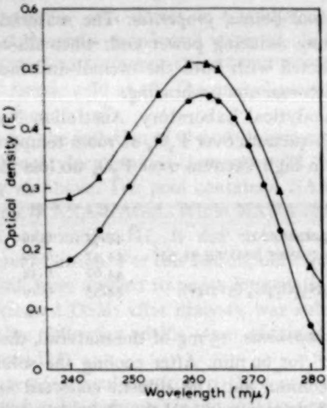

Fig. 1. U.V. absorption spectra in ethanol of authentic pyrrole-2-carboxylic acid and of the ether-soluble material obtained by alkali treatment of the acidic component of the prosthetic group of OSM. ●, authentic pyrrole-2-carboxylic acid; ▲, from prosthetic group.

Ratio of components: 1.68 mg of the material (dried over P_2O_5) was hydrolysed with 0.05 *N* H_2SO_4 at 80° for 60 min and the components of the hydrolysate separated as previously described. Determination of NANA in the lyophilized eluate gave a value of 0.99 mg (theory 1.01 mg). When the dried effluent was compared chromatographically with serial dilutions of NAGal, the amount of N-acetylhexosamine corresponded to the theoretical value of 0.73 mg within the error of the method[5] (± 10 %). The same results were obtained with α-neuraminidase as the hydrolysing catalyst.

Susceptibility to α-neuraminidase: When a sample of the material was treated with α-neuraminidase (RDE) and together with the control and reference substances chromatographed, complete cleavage of the prosthetic into NANA and NAGal was observed. As with acid hydrolysis of the material, the enzymic hydrolysis revealed a small amount of NATal.

Periodate oxidation: 3.6 mg of the material was oxidized at pH 4.0 with 0.01 *M* KIO_4 at 7°. The rate of periodate consumption is shown in Fig. 2.

Conversion by alkali of the prosthetic group to NANA-chromogen I and the enzymic cleavage of NANA-chromogen I: 5 mg of the material was treated with 1.0 ml 0.2 *N* Na_2CO_3 solution at 80° for 10 min. After removal of the cations with Dowex 50 (H^+) the resulting material was chromatographed before and after treatment with neuraminidase. Whereas the original material did not react with direct Ehrlich reagent in the cold, after alkali treatment a strong direct Ehrlich-reacting spot (in the cold) at R_F 0.20 was seen which on RDE treatment moved to R_F 0.61 (chromogen I standard R_F 0.61) concomitant with the appearance of an orcinol-reacting spot at R_F 0.15 (NANA standard R_F 0.15).

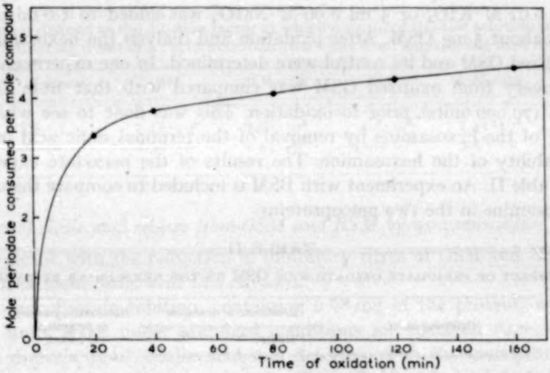

Fig. 2. Consumption of periodate by N-acetylneuraminyl-N-acetylgalactosamine. Conditions: 0.01 *M* KIO_4, pH 4, temperature 7°.

Identification of N-acetylneuraminyl-chromogen I formed by alkali treatment of OSM

The material obtained from pool III by selective elution from paper and purification on Dowex I (formate form) reacted even in high dilution immediately in the cold with Ehrlich reagent. It was found to be susceptible to α-neuraminidase and to behave chromatographically before and after enzymic treatment in the same manner as NANA-chromogen I produced by alkali treatment of the prosthetic group (see above).

The snow-white, non-crystalline material was dried over P_2O_5 in high vacuum at room temperature for several days; no weight was lost on further drying at 55°. Analysis:

Found: C, 46.47; H, 6.36; N, 5.32; ash, 0.84 %.

Calc. for $C_{19}H_{30}O_{13}N_2$ (494.45): C, 46.15; H, 6.12; N, 5.67 %.

1.33 mg of the dried material was hydrolysed with 0.05 *N* H_2SO_4 at 80° for 60 min and NANA after adsorption to and elution from Dowex 1-formate determined quantitatively. Found: 0.80 mg (theory 0.83 mg).

NANA-chromogen III, isolated from pool III as above and chromatographed, revealed a single orcinol- and direct Ehrlich-reacting spot at R_F 0.28; the RDE treated material gave rise to an orcinol-positive spot at R_F 0.15 (coincident with NANA standard) and a direct Ehrlich-positive spot at R_F 0.78 (coincident with chromogen III). The yield of NANA-chromogen III was insufficient for further analysis.

Identification of N-acetylneuraminic acid formed by alkali treatment of OSM

Crystalline NANA as obtained from pool I was dried over P_2O_5 in high vacuum and analysed (Microanalytical Laboratory, C.S.I.R.O., Melbourne).

Found: C, 42.6; H, 6.4; N, 4.6%.

Calculated for $C_{11}H_{19}O_9N$: C, 42.7; H, 6.2; N, 4.5 %.

Effect of periodate oxidation on the hexosamine recovery from OSM

10 ml 0.01 *M* KIO_4 or 3 ml 0.06 *M* $NaIO_4$ was added to 1.0 ml of a solution containing about 5 mg OSM. After oxidation and dialysis the hexosamine contents of the oxidized OSM and its control were determined. In one experiment the hexosamine recovery from oxidized OSM was compared with that from OSM treated with RDE (70,000 units) prior to oxidation. This was done to see what effect the unmasking of the hexosamine by removal of the terminal sialic acid may have on the oxidizability of the hexosamine. The results of the periodate experiments are shown in Table II. An experiment with BSM is included to compare the oxidizability of the hexosamine in the two mucoproteins.

TABLE II

EFFECT OF PERIODATE OXIDATION OF OSM ON THE HEXOSAMINE RECOVERY

Oxidizing agent	*Conditions of oxidation*		*Hexosamine recovery in percentage of control*
	Hours	*°C*	
KIO_4	3	10	55.6
KIO_4	24	10	15.2
KIO_4	24	20	8.7
$NaIO_4$	3	20	20.1
$NaIO_4$	6	20	16.9
$NaIO_4$	24	15	6.7
$NaIO_4$ after RDE treatment of OSM	24	15	6.1
$NaIO_4$ on BSM	24	20	29.2

Yield of N-acetylneuraminyl-NAGal and conversion products

In the experiment detailed under "*Preparation*", of the total NANA present, namely 1.16 g, 260 mg (= 22.5 %) was rendered dialysable by the first alkali treatment. Calculated as NANA, in pools I, II and III 178 mg was recovered in the form of the prosthetic group and compounds derived from it. On the second treatment, of 170 mg NANA made dialysable 130 mg NANA was recovered in the form of the prosthetic group and conversion products. Corresponding figures for NANA from the combined third and fourth treatments were 203 and 141 mg respectively. In these four treatments 55 % of the total NANA was detached from OSM in dialysable form; 71 % of this NANA was recovered in the prosthetic group and conversion products thereof. Further alkali treatments still yielded appreciable amounts of the prosthetic group thus indicating that the residual 45 % of NANA in the mucoprotein was also bound to NAGal.

Biological activities of OSM and BSM and their reduction by neuraminidase

The influenza virus haemagglutinin inhibitory titres of OSM and BSM with the PR8 and Lee strains as indicators and the reduction of these titres by neuraminidase (RDE) are shown in Table III.

TABLE III

INHIBITORY TITRES PER MILLIGRAM MUCOPROTEIN AND THEIR REDUCTION BY NEURAMINIDASE (RDE)

Conditions of test: Serial dilutions of mucoprotein in 0.25 ml volume mixed with 4 agglutinating doses of indicator virus in 0.025 ml and incubated at 4° for 60 min; then 0.025 ml of 5% fowl red blood cells added and pattern read after further 30 min at 4°.
Inhibitory titre is defined as the dilution factor at which 0.25 ml of the test solution prevents 3 out of 4 haemagglutinating doses of a particular indicator virus from aggregating fowl red blood cells.

Indicator	*OSM*	*OSM–RDE*	*BSM*	*BSM–RDE*
PR8 – i	$1.18\cdot10^6$	$6.86\cdot10^3$	$4.8\cdot10^4$	$1.42\cdot10^3$
Lee – i	$1.30\cdot10^5$	$2.44\cdot10^3$	$4.96\cdot10^2$	$3.48\cdot10$

Quantitation of sialic acid release from OSM and BSM by neuraminidase

Concomitant with the reduction in inhibitory titres of OSM and BSM on neuraminidase treatment sialic acid was released.

5.0 ml mucoprotein solution, containing 0.88 mg of the protein, were digested with 5000 units RDE under standard conditions for specified time, followed by exhaustive dialysis at 0°. Sialic acid was determined in the mucoprotein solution after dialysis and in appropriate controls. From OSM 76 % and 85 % of the total NANA were released after 7 h and 24 h respectively. The corresponding values for BSM were 68 % (7 h) and 76 % (24 h).

DISCUSSION

The dialysable material obtained from OSM by mild alkali treatment was separated into three distinct fractions: pool I (11 %), pool II (48 %) and pool III (41 %). The main constituent of each pool was isolated and analysed.

The compound from pool II consists of NANA, identified by conversion to pyrrole-2-carboxylic acid, and N-acetylgalactosamine, identified chromatographically and by oxidation to lyxose, in equimolecular proportion as shown by quantitation of the constituents. The elementary analysis conforms to $C_{19}H_{32}O_{14}N_2$ in agreement with the above composition. Cleavage by α-neuraminidase of the reducing saccharide into NANA and NAGal indicates α-ketosidic linkage of NANA to NAGal. The presence of this linkage with a deoxy and a carboxyl group vicinal to the ketosidic carbon atom accounts for the ease of hydrolysis by very dilute acid. The consumption by one mole of the compound (in the aldehydo form) of 4 moles IO_4^-, 2 moles per component, excludes substitution at carbon atoms 4 and 5 of NAGal. Conversion by mild alkali of the compound to NANA-chromogen I (a chromogen reacting in the cold with Ehrlich reagent) without elimination of chromogen I excludes substitution at C_3 and C_4 of NAGal. Therefore, 6-α-D-N-acetylneuraminyl-NAGal is the only structure compatible with the evidence presented (see Fig. 3); a more detailed discussion of this evidence has been given in the previous paper[8].

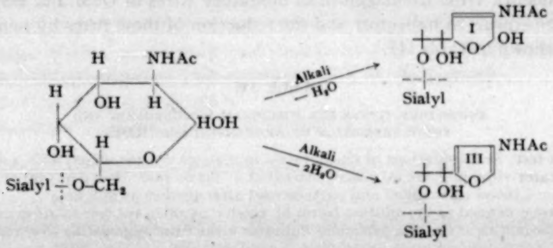

Fig. 3. Conversion by alkali of 6-α-D-N-acetylneuraminyl-N-acetylgalactosamine to NANA-chromogen I and NANA-chromogen III. Sialyl = N-acetylneuraminyl; --- bond susceptible to α-neuraminidase.

The statement that in the compound, isolated from pool II, the N-acetylhexosamine moiety is NAGal requires a qualification. As observed previously[8], small amounts of N-acetyltalosamine were released by dilute acid and by neuraminidase from the compound. The identification of NATal is based on (a) its colour reaction with the indirect Ehrlich reagent only, (b) its chromatographic identity with the substance formed in small quantity from authentic NAGal under the alkaline conditions of the release of the prosthetic group, (c) its oxidation to lyxose and (d) its identical R_F value with that reported in the literature. Its formation by epimerization of NAGal in alkaline medium is paralleled by the epimerization of N-acetylglucosamine to N-acetylmannosamine under similar conditions[21].

The high recovery from OSM of NANA in the form of NANA-NAGal and its conversion products on repeated mild alkali treatment taken together with the 1 : 1 ratio of NANA to NAGal in OSM and with the oxidizability of 93 % NAGal present in OSM provides strong evidence that 93 % of the total NANA is linked to carbon atom six of NAGal and that the disaccharide NANA-NAGal is the main prosthetic group of OSM. This means that in OSM as in BSM[8] and in the serum-type urine inhibitory mucoprotein[22] a considerable number of prosthetic groups are distributed along the protein chain. In the urinary inhibitor approximately 120 individual prosthetic groups are attached to the protein core[23]. In OSM (11.3% *N*) about one out of eight amino acids carries a prosthetic group.

The origin of NANA-chromogen I, the main component of pool III, is obvious from the convertibility by mild alkali of NANA-NAGal to NANA-chromogen I. NANA-chromogen I is identified as 6-α-D-N-acetylneuraminyl-anhydro-NAGal by elementary analysis, susceptibility to α-neuraminidase and by its origin. NANA-chromogen III, a minor component of pool III, is derived from the same source, as established previously[8].

The compound comprising pool I is NANA, identified by elementary analysis of the crystalline material. It may arise by two reaction mechanisms: (a) NANA (2 → 6) NAGal may be transformed under the impact of alkali to the ketoside of a

β-hydroxy-carbonyl compound; the glycosidic linkage of this type of compound is known to be alkali-sensitive[24]. (b) Since 7 % of the total hexosamine is unoxidizable, this fraction may be present in OSM in the form of NANA (2 → 3) NAGal which on alkali treatment would decompose by elimination to chromogen I, actually found in the dialysate of alkali treated OSM (see "*Preparation*"), and NANA.

The presence in our OSM preparation of very small amounts of sugars characteristic of the serum-type mucoprotein would suggest them to be the carbohydrate moiety of a contaminant rather than intrinsic components of OSM. If so, the contamination of glandular mucoproteins by small amounts of serum-type mucoproteins is assessed more sensitively by carbohydrate analysis than by electrophoresis.

The high biological activity of OSM is demonstrated by the observation that $0.85 \cdot 10^{-6}$ mg will inhibit 4 haemagglutinating doses of indicator virus (influenza strain PR8) compared with $0.21 \cdot 10^{-4}$ mg of BSM. The biological activity is strikingly reduced by α-neuraminidase, in OSM to 0.6 %, in BSM to 3.0 % of original activity. Similar observations have been reported by others[9,10]. The fact that neuraminidase removes from OSM and BSM 85 % and 76 % respectively of the total sialic acid present, and sialic acid only[25], strongly suggests the terminal sialic acid in inhibitory mucoproteins to be the main anchoring group for the influenza virus particle.

The relationship between chemical structure and inhibitory activity of OSM and of BSM will be treated separately[26].

ACKNOWLEDGEMENTS

We are greatly indebted to Dr. S. Fazekas de St. Groth for testing the biological activity of our mucoprotein preparations, to Dr. J. E. Fildes for the elementary analyses and to Drs. Ada and French for supply of RDE.

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HYDROLYSIS OF LECITHINS BY VENOM PHOSPHOLIPASE A

I. STRUCTURE OF THE ENZYMICALLY FORMED LYSOLECITHINS

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SUMMARY

Rat-liver $[^{32}P]$lecithin was hydrolyzed by snake venom phospholipase A to give quantitative yield of $[^{32}P]$lysolecithins. The lysolecithins were oxidized in buffered aqueous solutions in the pH range 2.8 to 7.0, with bromine or permanganate to yield a keto-lysolecithin derivative. The extent and rate of oxidation is pH dependent. Bromine oxidation is more rapid at pH 6.8 than at pH 5.5 or 2.8. Permanganate oxidation is rapid at pH 5.5 but very slow at pH 6.8. During the oxidation partial concomitant hydrolysis occurs.

The keto-lysolecithin can be separated and measured by paper chromatography and thus permits the determination of the α-lysolecithin isomer. The amount of this isomer was found to vary between 54–94 % depending on the pH at which the bromine oxidation is carried out. Definitive evidence for the formation of the lysolecithinic acid has not yet been obtained.

Passage of lysolecithin through silicic acid causes ester migration from the beta to the alpha position of glycerol. This is reflected by an increase in the keto-lysolecithin upon bromine oxidation.

A novel reaction occurs when lysolecithin is treated with bromine in chloroform or methanol. Brominolysis rather than oxidation takes place with the formation of lysophosphatidic acids and choline.

A discussion of these findings is presented.

INTRODUCTION

In a previous paper[1] it was shown that permanganate oxidation of enzymically produced lysolecithins yielded two products, one of which was a ketone derivative. Acid hydrolysis of the oxidized lysolecithins gave rise to at least four water soluble phosphate compounds. These data provided evidence that the lysolecithins were a mixture of alpha and beta isomers and consequently that the venom phospholipase A hydrolyzes both the alpha and beta fatty acid ester groups on lecithin. Supporting evidence that the phospholipase A can hydrolyze the beta fatty acid ester linkage was obtained by studies on the hydrolysis of choline-plasmalogens[2]. The lysoplasmalogens which were formed were reduced with hydrogen and hydrolyzed to yield α-glycerol ethers.

Since permanganate oxidation did result in appreciable hydrolysis of the lysolecithins even in buffered solutions a search was made for another oxidant. Bromine was found to be effective[3]. Studies with this oxidant will be reported in this paper. By use of [^{32}P]lysolecithin the rate of oxidation at various pH values was investigated. Both bromine and permanganate oxidation were found to be pH dependent. The studies to date have shown that bromine oxidizes the secondary hydroxyl group on the lysolecithin to yield a keto-derivative. Evidence for the oxidation of the primary hydroxyl group to yield a lysolecithinic acid has not yet been obtained. As is the case with permanganate, during bromine oxidation some hydrolysis of the lysolecithins occurs.

In contrast to the oxidation which occurs in buffered aqueous solutions of bromine, in non-aqueous solvents a novel type of brominolysis takes place. Thus lysolecithin undergoes brominolysis in either chloroform or methanol to yield choline and lysophosphatidic acids[3,4].

The fatty acids which were liberated by hydrolysis by the venom phospholipase were also investigated. As shown by others[5,6] the liberated fatty acids are predominantly unsaturated. However, we have observed that the venom enzyme shows chain length preference. Hence when two different fatty acids are present on the lecithin, the phospholipase removes the longer chain acid. The latter experiments will be reported in another paper[7].

METHODS AND REAGENTS

Preparation of lecithins and lysolecithins

The lecithins of egg and the [^{32}P]lysolecithin of rat liver were prepared as described previously[1]. The lecithins of pig heart were made by the procedure reported elsewhere[8]. The snake venoms (*Crotalus adamanteus* and *Naja naja*) were obtained from the Ross Allen Reptile Institute, Silver Springs, Florida. The hydrolysis of the lecithins by the venoms was carried out in ether solution in a manner described previously[1].

The lecithins of egg and pig heart and the fatty acids liberated by venom hydrolysis of rat-liver lecithin were reduced with hydrogen over platinum oxide catalyst in ethanol at 50 lbs. pressure at room temperature.

Bromine and permanganate oxidation of the lysolecithins in buffered aqueous solution

The oxidation of the lysolecithins was carried out as given in the legends to the figures.

Chromatography of the water soluble phosphate esters and choline

The water soluble phosphate compounds which were liberated during the oxidation of the lysolecithins or which were liberated by 1 *N* HCl hydrolysis of the oxidized and parent lysolecithins were qualitatively analyzed by paper chromatography as described previously[1]. Choline wasqualitatively analyzed by chromatography on Whatman No. 1 filter paper in the same solvents used for the phosphate compounds and in a solvent consisting of *n*-butanol–ethylene glycol–water (4:1:1). The phosphate esters were detected by the HANES AND ISHERWOOD reagent[9] or by autoradiography. Choline was detected by the phosphomolybdate-stannous chloride test[10].

RESULTS

The autoradiograms in Fig. 1 demonstrate the qualitative behavior of the [^{32}P]rat-liver lysolecithin in various aqueous solutions. In all systems A–F the lysolecithin which was used had been passed through a column of silicic acid. The control system A represents lysolecithin dissolved in distilled water. Essentially no hydrolysis occurs over a period of 2 days at room temperature. The same is true for lysolecithin dissolved in acetate buffer pH 5.5 or in phosphate buffer pH 6.8 and 7.4. The lysolecithin appears as a single spot and practically no radioactivity is seen at the origin (spot 0).

The lysolecithin dissolved in 0.05 *N* HCl, shown in system B, has undergone appreciable hydrolysis. The origin spot No. 0 in this case represents either glycerol phosphate or/and glycerylphosphorylcholine. The extent of hydrolysis is 23 and 34 %

Fig. 1. Autoradiograms showing the behavior of [^{32}P]lysolecithin in various aqueous solutions with and without bromine. The lysolecithin was obtained by hydrolysis of [^{32}P]rat-liver lecithin with *Naja naja* venom. The lysolecithin in these experiments had been passed through silicic acid. The lysolecithin (4.2 mg) was dissolved in the following solutions: (A) 1.0 ml of distilled water (this served as the control); (B) 1.0 ml of 0.05 *N* HCl; (C) 1.0 ml of distilled water plus 4 µl of liquid bromine; (D) 1.0 ml of 0.2 *M* phosphate buffer pH 7.4 plus 4 µl of liquid bromine (the measured pH of this solution after addition of the bromine was 7.0); (E) 1.0 ml of 0.2 *M* acetate buffer pH 5.5 plus 4 µl of liquid bromine (the measured pH of this solution after addition of bromine was pH 5.1); and (F) 1.0 ml of 0.2 *M* phosphate buffer pH 6.8 plus 4 µl of liquid bromine (the measured pH of this solution after addition of bromine was 6.4). After 24 h 15 µl of each solution were pipetted on silicic acid impregnated paper and chromatography was carried out in a solvent consisting of diisobutyl ketone–acetic acid–water (40:25:5)[11]. The length of the chromatographic run was 26 h at 23°. The autoradiograms were done on No-Screen X-Ray film and required 5 days exposure. Spot 0 = water soluble phosphate esters which remain at the origin and form as a result of hydrolysis of the lysolecithins. Spot 1 = the parent lysolecithin and Spot 2 = keto-lysolecithin which forms as a result of bromine oxidation. Both spots 1 and 2 gave a positive test for choline[10,11] but only spot 2 gave a positive ketone test with dinitrophenylhydrazine[11]*.

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* The test for ketone is carried out as follows. The chromatogram is washed in distilled water for 15 min and then dried. The dry chromatogram is passed several times through a solution of 2,4-dinitrophenylhydrazine in 3 *N* HCl[11]. The chromatogram is hung for 3 min and then immersed in distilled water to wash out the excess reagent. Three 10-min washes are generally sufficient. A positive test is indicated by a yellow or yellow orange spot which shows up as a dark absorbing spot under u.v. light (366 mµ). This latter property is essential since some lipids may adsorb the dinitrophenylhydrazine and appeared yellow but do not absorb in the u.v. region.

after 24 and 48 h respectively. In system C is shown the behavior of lysolecithin in unbuffered bromine water (pH about 2.8). The extent of oxidation under these conditions is small (6–10 %) as evidenced by the small amount of keto-lysolecithin which forms (spot 2). The keto function is demonstrated by the dinitrophenylhydrazine test. However appreciable hydrolysis of the lysolecithin occurs due to the low pH. The extent of hydrolysis in system C is 29 and 38 % after 24 and 48 h respectively.

System D shows the bromine oxidation of lysolecithin in phosphate buffer pH 7.0*. It can be seen that the keto-lysolecithin, spot 2, is the major component, thus demonstrating that the oxidation of the lysolecithin was nearly complete and that the lysolecithin was predominantly the alpha-isomer. Hydrolysis also occurred during the oxidation since water soluble phosphate compounds appear at the origin. These latter compounds were not identified but at least three components were found to occur in this origin spot.

The bromine oxidation of the lysolecithin in acetate buffer pH 5.1* and in phosphate buffer pH 6.4* are depicted in systems E and F. In both cases the keto-lysolecithin is produced but the oxidation had not proceeded to completion. It is evident that in all cases where oxidation occurs in buffered solution hydrolysis also takes place, while in the buffer controls at about the same pH but not containing bromine no hydrolysis can be detected.

The chromatographic data in Fig. 2 show both the qualitative and quantitative aspects of the reaction of lysolecithin in buffered aqueous solutions containing bromine or permanganate. Also shown for sake of comparison is the behavior of lysolecithin in a non-aqueous solvent (chloroform) containing bromine (system G). The effect of silicic acid on the structure of the lysolecithin is demonstrated by the data. The NLL lysolecithin represents the normal lysolecithin which was not subjected to silicic acid treatment. The SLL lysolecithin represents the lysolecithin after passage through a column of silicic acid. The lysolecithin (25 mg) was dissolved in chloroform and put on a 10 g column of silicic acid and eluted with methanol. The total time that the lysolecithin was exposed to the silicic acid was 6 h.

It should be pointed out that in order to obtain quantitative data from the chromatograms, the origin spots must be cut off before the chromatograms are dipped in the Rhodamine 6G solution. Immersion of the chromatograms in the aqueous dye for 1–3 min does not effect an appreciable loss of material from the lysolecithin or the keto-lysolecithin spots but does effect an 80–90 % loss of material from the origin spots. The numbers in each spot in Fig. 2 represent the amount of radioactivity in these components. These activities are calculated as the per cent of total radioactivity applied to the paper in each strip. The extent of oxidation by bromine as measured by the appearance of the keto-lysolecithin (spot 2) was greater at pH 6.4* (systems C and D) than at pH 5.1* (systems A and B). The data show that after silicic acid treatment the amount of keto-lysolecithin is increased, in particular at pH 6.4 (and more so at pH 7.0* as seen in Fig. 1 system D). Therefore during chromatography on silicic acid beta to alpha acyl migration of the fatty acid occurs. A similar finding has been reported for monoglycerides[12,13].

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[*] The pH of the buffers which were used was 5.5, 6.8 and 7.4. However the pH after addition of bromine to these buffers (and hence the pH at which the reaction occurred) was 5.1, 6.4 and 7.0 respectively. Permanganate had no measurable effect on the pH of the buffers.

In Table I are given data showing the amount of keto-lysolecithin and the lysolecithin which resists oxidation when egg lysolecithin is oxidized with bromine at pH 5.1 and 6.4. This experiment confirms the work on rat-liver lysolecithin since in both cases after treatment of the lysolecithins with silicic acid the amount of keto-lysolecithin increases. Therefore acyl migration of the fatty acid occurs with both egg and rat-liver lysophosphatides when chromatographed on silicic acid.

The oxidation of lysolecithin by permanganate at pH 5.5* is shown in system E.

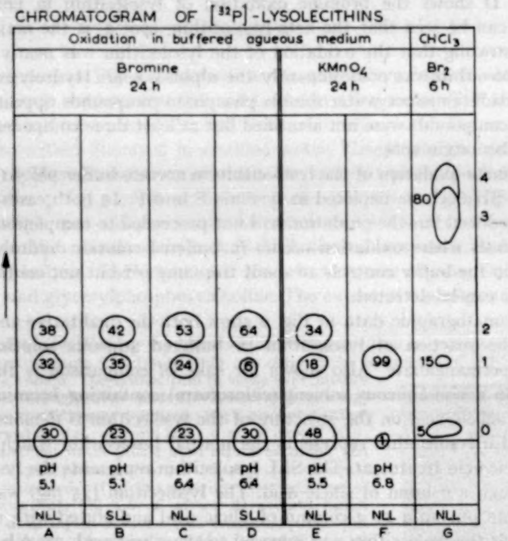

Fig. 2. Chromatograms showing the behavior of [^{32}P]lysolecithin in various buffered solutions toward bromine and permanganate and in chloroform solution toward bromine. NLL = the normal lysolecithin which had not been exposed to silicic acid and SLL = the same lysolecithin which had been passed through a column of silicic acid as explained in the text. 2 mg of either the NLL or SLL lysolecithin were dissolved in the following solutions: (A) and (B) – 0.35 ml of water plus 0.35 ml of 0.2 *M* acetate buffer pH 5.5* plus 5 microliters of bromine; (C) and (D) 0.35 ml of distilled water plus 0.35 ml of 0.2 *M* phosphate buffer pH 6.8* plus 5 μl of bromine; (E) 0.35 ml of distilled water plus 0.35 ml of 0.2 *M* acetate buffer pH 5.5* plus 2 mg of solid KMnO$_4$; (F) 0.35 ml of distilled water plus 0.35 ml of 0.2 *M* phosphate buffer pH 6.8* plus 2 mg of KMnO$_4$; and (G) 0.5 ml of chloroform plus 3 μl of bromine. After 24 h at room temperature for systems (A)–(F) and after 6 h for system (G) 20-μl aliquots were taken for chromatographic analysis as described in Fig. 1. Spot 0 = water soluble phosphate esters, spot 1 = lysolecithin, spot 2 = keto-lysolecithin, and spot 3 plus 4 = lysophosphatidic acids. The origin spots were cut off and the radioactivity in each was determined. The remaining part of the chromatogram was stained with Rhodamine 6G[11] and the lipid spots observed and marked under u.v. light. Spots 1 and 2 stained yellow with Rhodamine 6G and gave a positive test for choline[10,11] whereas spots 3 and 4 stained blue with Rhodamine 6G and gave a negative test for choline. Only spot 2 gave a positive ketone test with 2,4-dinitrophenylhydrazine[11]**. After the chromatograms were dry the spots were cut off and the radioactivity in each was determined. The numbers in or by each spot refer to the per cent of total radioactivity in each component (*i.e.* the per cent of the total radioactivity in the 20-μl aliquot which was used for chromatography).

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* See footnote on page 527.

** See footnote on page 526.

TABLE I

EFFECT OF SILICIC ACID ON THE STRUCTURE OF EGG LYSOLECITHIN

These analyses were performed on the bromine oxidation products of reduced lysolecithins obtained by venom hydrolysis of reduced egg lecithin. The bromine oxidation was carried out at pH 5.1 and 6.4 in the same manner as described in Fig. 2. Aliquots of the reaction mixture were taken for paper chromatographic analysis[11]. The unreacted lysolecithin spot (spot 1) and the keto-lysolecithin spot (spot 2) were combined from several chromatograms, eluted with methanolic-HCl and the lipid P was done on the eluates in a manner described previously[11]. The origin spots were not analyzed in this experiment. Hence the data are given as the fraction of the total lipid P which was found in the lysolecithin and keto-lysolecithin.

Sample	*Percent of total lipid P*		*pH*
	Keto-lysolecithin	*Lysolecithin*[*]	
Egg lysolecithin			
Untreated	72	28	5.1
Passed through silicic acid	80	20	
Untreated	84	16	6.4
Passed through silicic acid	91	9	

[*] The lysolecithin corresponds to spot 1 in Figs. 1 and 2. This lysolecithin resists oxidation.

We have reported details of this oxidation previously[1]. In the case of permanganate however, the extent of hydrolysis is much higher than when bromine is used at the same pH[*]. The unexpected observation was made that the permanganate oxidation of lysolecithin is completely abolished by raising the pH to 6.8 (system F). No keto-lysolecithin is formed after 24 h at room temperature. The buffer in this case contained phosphate rather than acetate.

In contrast to the oxidation of lysolecithin in aqueous solution, in non-aqueous solvents a novel reaction occurs. Thus in chloroform or methanol the lysolecithin undergoes brominolysis to yield choline and lysophosphatidic acids (spots 3 and 4 of system G). Details of this reaction will be reported elsewhere[4].

The use of $[^{32}P]$lysolecithin coupled with the quantitative paper chromatographic method permitted a study of the rate of bromine oxidation at various pH levels. In Fig. 3 are shown the results of an experiment at pH 5.1. The rate of disappearance of lysolecithin (curve 3), the rate of appearance of the keto-derivative (curve 2) and the rate of formation of water soluble phosphate compounds at the origin (curve 1) are given. The oxidation is complete after 24 h under these conditions. There is a slow increase in the production of water soluble phosphate compounds with a concomitant decrease in the amount of keto-lysolecithin after this latter compound has attained a maximum value.

In Fig. 4 are shown the curves for the rate of disappearance of lysolecithin at 3 different pH levels. The rate and hence the extent of oxidation of lysolecithin increases with increasing pH.

The rate curves for the concomitant production of the keto-lysolecithin are given in Fig. 5. It is again evident that the rate of oxidation is most rapid at pH 7.0 but that the keto-lysolecithin which is formed undergoes hydrolysis. An analysis of the water soluble phosphate compounds at the origin demonstrated a parallel increase

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[*] In our previous work[1] the failure to observe appreciable radioactivity at the origin was due to this phenomenon.

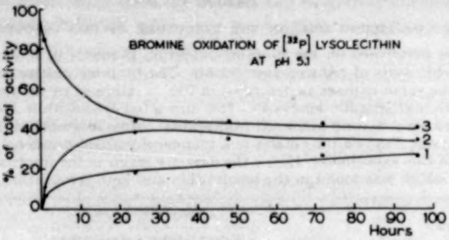

Fig. 3. Bromine oxidation of [^{32}P]lysolecithin in acetate buffer pH 5.1. The oxidation was carried out as given in system (E) in Fig. 1. At the time intervals indicated 15 μl aliquots were removed for paper chromatographic analysis. Curve 1 represents the water soluble phosphate compounds at the origin, curve 2 represents the keto-lysolecithin (spot 2) and curve 3 represents the lysolecithin (spot 1). The acetate buffer which was added was pH 5.5 but the actual pH of the reacting solution was pH 5.1.

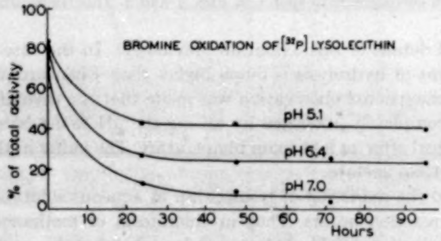

Fig. 4. Bromine oxidation of [^{32}P]lysolecithin at various pH. The oxidation was carried out as given in systems (D), (E) and (F) in Fig. 1. At the time intervals indicated 15-μl aliquots of the solution were removed for paper chromatographic analysis. The curves show the rate of disappearance of lysolecithin (spot 1). In Fig. 5 are given the curves for the rate of formation of the keto-lysolecithin (spot 2).

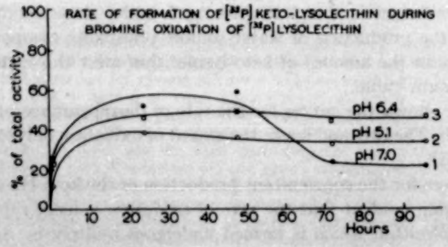

Fig. 5. Rate of formation of keto-lysolecithin during bromine oxidation of [^{32}P]lysolecithin a) various pH. The oxidation of lysolecithin was carried out as given in systems (D), (E) and (Ft in Fig. 1. The spots were taken from the same chromatograms used for the data shown in Fig. 4. In this case the radioactivity in the keto-lysolecithin (spot 2) was measured with time.

which accompanied the decrease in the keto-derivative. It is also evident that the rate of formation of the keto-lysolecithin parallels the rate of disappearance of lysolecithin (compare the curves in Figs. 4 and 5).

The products formed during the reaction of lysolecithin with bromine were also investigated. The lysolecithin and keto-lysolecithin were identified by paper chromatography and chemical analysis. These data are summarized in Table II. Both the lysolecithin and keto-lysolecithin contain P and choline and give a yellow staining reaction with Rhodamine 6G[11]. The keto-lysolecithin, however, has a higher R_F value than lysolecithin and it alone gives a positive test with dinitrophenylhydrazine[11].

TABLE II

CHARACTERIZATION OF THE PRODUCTS OBTAINED AFTER BROMINE OXIDATION OF [^{32}P]RAT-LIVER LYSOLECITHIN

The spot tests were done on chromatograms similar to those shown in Figs. 1 and 2. These spots were combined from 18 chromatograms. The phosphate esters were determined by hydrolysis of the material in spots 1 and 2 with 1 *N* HCl for 1 h at 100° and chromatography in several solvents[1]. The choline released was determined qualitatively by paper chromatographic analysis of the same acid hydrolysate used for the phosphate esters. Choline was chromatographed on Whatman No 1 filter paper in a solvent consisting of *n*-butanol–ethylene glycol–water (4:1:1) or *n*-propanol–ammonia–water (60:30:10).

*Compound**	*Spot tests**				*Phosphate ester**	*Choline released**
	Choline	*DNPH*	*Rh.6G*	*^{32}P*		
Spot-1 lysolecithin	+	—	Yellow	+	GP	++++
Spot-2 keto-lysolecithin	+	+	Yellow	+	PC + unid.	+
Spots 3 + 4 lysophosphatidic acid	—	—	Blue	+	GP	—

* GP = glycerol phosphate, PC = phosphorylcholine, unid. = unidentified phosphate compound. DNPH = test with 2,4-dinitrophenylhydrazine, Rh.6G = Rhodamine 6G, ^{32}P = radioactivity of labeled phosphate as determined by autoradiography. (See legend to Figs. 1 and 2).

Furthermore, the keto-lysolecithin yields a different phosphate compound on acid hydrolysis than does lysolecithin. The major phosphate ester obtained by acid hydrolysis of the keto-derivative has the chromatographic mobility of phosphorylcholine in three different solvent systems. The minor phosphate compound which is obtained from the keto-compound has not been identified. The phosphate ester obtained by acid hydrolysis of the lysolecithin (spot 1) which resists oxidation has the identical mobility as glycerolphosphate in three different solvents. Furthermore, free choline is liberated by acid hydrolysis of spot 1 but only a very small amount of free choline is obtained by acid hydrolysis of the keto-compound spot 2. We originally considered[1] the possibility that the slower spot 1 which resists oxidation might be lysolecithinic acid. This, however, is not the case since the evidence clearly shows that this material is the parent lysolecithin. It is noteworthy that lysolecithinic acid would not be expected to have the same mobility as lysolecithin, should stain blue with Rhodamine 6G (as do other acidic phosphatides such as inositol phosphatide, phosphatidylserine, and phosphatidic acids[11]) and would yield phosphoglyceric acids on acid hydrolysis.

The behavior of lysolecithin in 0.05 *N* HCl at room temperature is of further interest. As previously noted by HANAHAN[14] we have consistently found that when lysolecithin is dissolved in acid solution of this normality the solution slowly gels.

In our studies the gelling required between 10–24 h. However, appreciable hydrolysis (23–34 %) of the lysolecithin occurs (Fig. 1, system B). Subsequent bromine oxidation of these solutions (after the pH had been brought up to 5.1 to 7.0) demonstrated that acyl migration had not occurred, since the yield of keto-lysolecithin was not increased. Indeed, the yield of keto-lysolecithin showed a small decrease. Hence the gelling phenomenon does not appear to be due to beta to alpha acyl isomerization as HANAHAN proposes. We feel that the gelling may be due to one or more of the following: (a) a physical effect due to the liberation of fatty acids and water soluble phosphate compounds (b) an effect due to the cationic form of lysolecithin which would predominate in acid solution and (c) phosphate migration from the alpha to the beta position of glycerol to form lysolecithins having phosphorylcholine attached to the C-2 position. With regard to point (c) the properties of such synthetic "beta" lysolecithins (*i.e.* with respect to the position of attachment of the phosphorylcholine) reported by BAYLIS *et al.* is noteworthy[15]. These workers state that aqueous solutions of their synthetic "beta" lysolecithins form gelatinous solutions.

In order to test the effect of enzyme concentration and time of exposure of the lipides to the venom, egg lecithin was treated with a much smaller amount of venom (3 μg of *Naja naja* venom per mg of egg lecithin) for 40, 80, 120, and 180 min. The lysolecithins obtained in each case were oxidized by bromine in phosphate buffer pH 6.4. In all cases the keto-lysolecithin was obtained in essentially the same yield. These experiments make it unlikely that snake venom contains an active acyl isomerase which causes a migration of the fatty acid from the beta to the alpha position of glycerol.

DISCUSSION

Some of the presented data are not in accord with the work of others[16–18]. The apparent discrepancy might be attributed to a number of factors some of which are as follows: (a) difference in enzyme preparation (b) difference in experimental conditions (c) acyl migration either during enzyme hydrolysis, during the oxidation of the lysolecithins or during the isolation and purification of the lysolecithins, (d) hydrolysis of the lysolecithins during oxidation. Some of these considerations have been pointed out previously[1].

The venom (cobra and rattlesnake) used in these experiments were the same as those employed by HANAHAN[16]. The venom used by GRAY[18] was obtained from water moccasin. Isomerase activity may conceivably play a role in this study.

Acyl migration during oxidation of the lysolecithins may occur but this possibility is unlikely in the case of bromine at pH 5.1 to 7.0 since the rate of lysolecithin disappearance levels off even in the presence of a large excess of oxidant. Furthermore, after passage of the lysolecithin through silicic acid a significant change in the amount of keto-lysolecithin was detected. These results would not be obtained if acyl migration occurred during the oxidation.

The experimental data demonstrate that both permanganate and bromine are capable of oxidizing lysolecithin but that the oxidation is pH dependent. It is important to note that there is a concomitant hydrolysis during the oxidation and that this hydrolysis is greater with permanganate than with bromine at pH 5.1–5.5. Hence it was concluded that the solution of this problem, in particular with respect to the structure of the lysolecithins, is most easily accomplished by the direct measurement of the rate of formation of the keto-lysolecithin or the rate of disappearance of the lysolecithin rather than by the analysis of the water soluble phosphate esters or other degradation products which result by subsequent acid hydrolysis.

Although the yield of keto-lysolecithin is pH dependent it can be inferred that the amount of α-lysolecithin in the mixture of lysolecithins which are produced by venom hydrolysis is between 54–92 %. The reason why the remaining lysolecithin (spot 1) resists hydrolysis is not clear. The existence of beta lysolecithins (*i.e.* lysolecithins with the fatty acid on the C-2 position of glycerol) is supported by the finding that silicic and treatment results in acyl migration and increases the content of the alpha-isomer. The evidence therefore demonstrates that the phospholipase can hydrolyze the beta-linked fatty acid. Confirmatory evidence for this finding comes from our previous work with heart muscle choline plasmalogens where it was demonstrated that reduction and subsequent hydrolysis of the enzymically formed lysoplasmalogens yielded α-glycerol ethers[2*].

The reason why the yield of keto-lysolecithin increases with increasing pH is not understood. This effect may be due to the nature of the oxidant. Hence at lower pH levels the oxidant may be molecular bromine whereas at the higher pH level the oxidant may be the hypobromite ion or possibly molecular oxygen which forms as a result of decomposition of hypobromite. Oxidation at pH higher than 7.4 was not investigated because of the lability of ester groups in alkaline solution.

Since this study demonstrates that passage of lysolecithin through silicic acid brings about acyl migration, any work on the structure of these lipids must take this finding into account. The apparent discrepancy between our work and that of GRAY may in part be due to this phenomenon. Isomerization of lipids on silicic acid has also been demonstrated by BORGSTROM[12] and HIRSCH AND AHRENS[13]. These workers were able to show the production of 1-monoglycerides when 2-monoglycerides were passed through silicic acid.

The analysis of the water soluble phosphate compounds obtained by acid hydrolysis of the reaction mixture after oxidation of the lysolecithins presents a difficult problem since appreciable hydrolysis occurs during the oxidation and hence several phosphate compounds are formed. The number of water soluble phosphate esters produced in this manner would be decreased if the intermediate oxidized lysolecithins were first isolated and separated, and then hydrolyzed. In this case, however, the yield of each product must be considered in the evaluation of the quantitative significance of such findings. In our hands it has been simpler and more direct to analyze the oxidation product (keto-lysolecithin). These considerations may help explain the apparent discrepancy between our work and the work reported by others[16–18].

ACKNOWLEDGEMENT

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[*] DRS. A. TATTRIE and M. KATES at the National Research Council, Ottawa, Canada have obtained further data by an independent method that the snake venom phospholipase A liberates preferentially the β-linked fatty acid of egg lecithin (personal communication). This work is now in press (J. Lipid Research).

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HYDROLYSIS OF LECITHINS BY VENOM PHOSPHOLIPASE A

II. FATTY ACID CHAIN LENGTH PREFERENCE OF THE ENZYME

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SUMMARY

Phospholipase A of snake venom shows chain length preference with respect to the hydrolysis of lecithin fatty acids. With pig heart, egg, and rat-liver lecithins the C-22, C-20 and C-18 acids are hydrolyzed in preference to the C-16 acids. It also appears that the C-22 and C-20 acids are hydrolyzed in preference to both the C-16 and C-18 acids.

INTRODUCTION

In a previous paper[1] the structure of the enzymically produced lysolecithins was investigated. Evidence was provided that snake venom phospholipase A hydrolyzes either the alpha or beta linked fatty acid on lecithin to yield a mixture of alpha and beta lysolecithins. However, the beta linked fatty acid is preferentially removed in some cases. This paper will deal with the analysis of the fatty acids which are hydrolyzed by the enzyme. With pig heart, egg, and rat-liver lecithins the C-22, C-20 and C-18 acids are hydrolyzed in preference to the C-16 acids. It also appears that the C-20 and C-22 acids are removed in preference to both the C-16 and C-18 acids.

METHODS AND REAGENTS

The experimental procedures for the preparation of the lecithins and the hydrolysis of the lecithins by the phospholipase A are reported in previous papers[2,3]. The fatty acids were analyzed by paper chromatography on mineral oil impregnated paper[4]. The solvent was acetic acid–water (9:1)*. In the case of rat-liver lecithin the fatty acids were analyzed before and after reduction with hydrogen. The reduction was carried out in methanol over platinum oxide at 50 lbs. pressure. The lecithins and lysolecithins were also hydrolyzed with 1 *N* HCl for 1 h and the fatty acids obtained by ether extraction in the usual way were analyzed by paper chromatography.

The fatty acids on the chromatograms were detected in the following manner: The chromatograms were dried in air for several hours, immersed in a 0.001 % solution of Rhodamine 6G for about 2 min, and then washed by immersing in distilled water for 15 min. The chromatograms were dried in air and observed under u.v. light (366 mμ). The fatty acids appear as yellow staining spots. These spots can be markedly intensified by passing the chromatogram several times through a 0.2 % aqueous alkaline solution of brom thymol blue and washing off the excess dye with water. The fatty acids were also detected by the procedures given by ASHLEY AND WESTPHAL[5]. The unsaturated acids were detected by dipping the chromatogram in a 1 % solution of potassium permanganate and washing off the excess permanganate. Brown spots appear where unsaturated fatty acids occur.

RESULTS AND DISCUSSION

The chromatographic analysis of the fully reduced (saturated) fatty acids of egg lecithin, egg lysolecithin, pig heart lysolecithin, and the fatty acids liberated by phospholipase A from the reduced egg and pig heart lecithin are shown in Fig. 1. Also included for sake of reference is a standard mixture of myristic (C-14), palmitic (C-16), and stearic (C-18) acids. The fatty acids of reduced egg lecithin were found to be mainly C-18 and C-16 and smaller amounts of C-20 and C-22. The fatty acids of egg phosphatides are given elsewhere[6]. The egg lysolecithin fatty acids were found to be C-16 and C-18 with the former in higher amount. No C-20 and C-22 acids were detected in the lysolecithin**. Hence the higher chain fatty acids are completely removed by the phospholipase A. The fatty acids released by Crotalus adamanteus venom by hydrolysis of the reduced egg lecithin are predominantly C-18 and smaller amounts of C-20 and C-22. No detectable amounts of C-16 acids were released by the venom**.

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* Whatman No. 1 filter paper was impregnated by passing once through a 10 % solution (v/v) of liquid petrolatum in benzene. The solvent system was acetic acid–water (9:1) saturated with liquid petrolatum. Chromatography was carried out by the ascending technique in cylinders (6″ dia. × 18″ in height) which were lined internally with Whatman No. 1 filter paper. Chromatography was carried out at 23° and required 24–30 h. The fatty acids were dissolved in chloroform at a concentration of 20–30 μg/10 μl. 10 and 20 μl were used for chromatography.

** These acids may occur to the extent of a few percent and not be detected by these procedures.

In the case of reduced pig heart lecithin (which was a mixture of the diester lecithin and plasmalogen in the ratio of 3 : 2) the fatty acids of the lysolecithins are a mixture of C-16, C-18, and C-20 and a small amount of C-22. The long chain mono-ether of glycerolphosphoric acid (GP-ether) also occurs in this fraction. The fatty acids released by the venom are predominantly C-20, a lesser amount of C-18, and a very small amount of C-22. No detectable amounts of C-16 acids were released by the venom**.

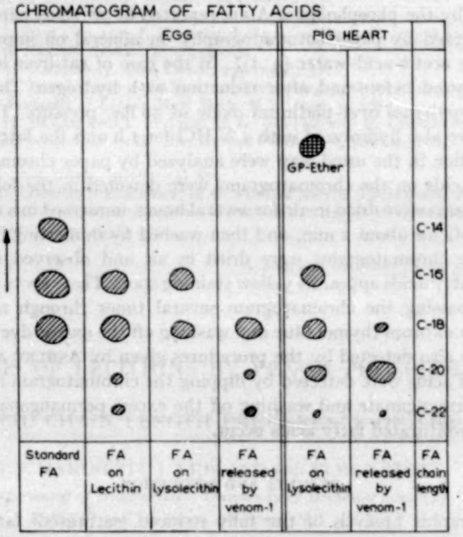

Fig. 1. Chromatograms of the fatty acids of reduced egg and pig heart lecithins and their corresponding enzymically produced lysolecithins. Chromatography was carried out as explained in the text. The standard fatty acids are myristic (C-14), palmitic (C-16) and stearic (C-18). GP-ether = the long chain ether of glycerolphosphate. This ether results by hydrolysis of the reduced plasmalogens which were found in the pig-heart lecithin. Venom —1 = *Crotalus adamanteus* venom. FA signifies fatty acid.

The paper chromatographic analysis of the fatty acids of rat-liver lysolecithin, and the fatty acids released by both *Naja naja* and *Crotalus adamanteus* are shown in Fig. 2. In this case the venoms were allowed to act on the unreduced lecithins. The fatty acids released by the venom are predominantly unsaturated C-18, C-20 and C-22. These acids gave a strong positive test with the permanganate reagent. The fatty acids of the rat-liver lysolecithins are predominantly saturated C-16 and C-18 (they fail to react with permanganate). When the fatty acids released by the venom were hydrogenated it became apparent that these acids were predominantly C-20 but that smaller amounts of C-18 and C-22 acids also occurred. Although the venoms liberated the C-20 acids in highest amount, in the case of *Naja naja* less C-18 and more C-20

** See footnote on page 535.

acids were released than was so with *Crotalus adamanteus*. Hence with three different lecithin preparations no detectable amount of C-16 acids were liberated by the phospholipase.

The fatty acids of the keto-lysolecithin produced by bromine oxidation[1] of egg lecithin and the fatty acids of the lysolecithin which resists oxidation by bromine were also investigated. Both lyso-compounds were found to contain C-16 and C-18 acids. The C-16 acids predominated. It is apparent from these studies that egg lecithin is heterogeneous with respect to the fatty acids and it is unlikely that lecithins containing both fatty acids of the same type (*i.e.* distearyl, dipalmityl, dioleyl, etc.) occur in egg in appreciable amount.

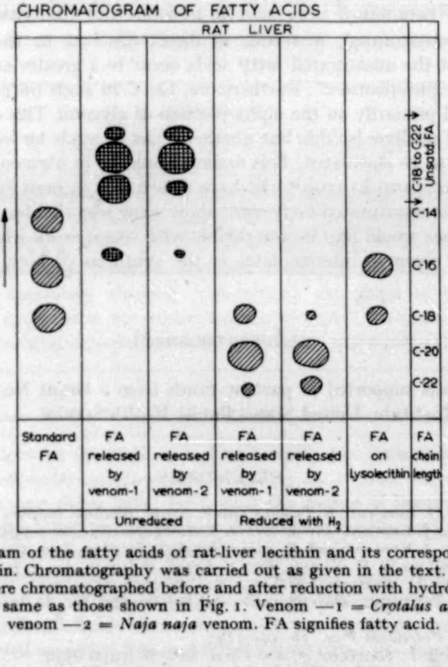

Fig. 2. Chromatogram of the fatty acids of rat-liver lecithin and its corresponding enzymically produced lysolecithin. Chromatography was carried out as given in the text. The fatty acids of rat-liver lecithin were chromatographed before and after reduction with hydrogen. The standard fatty acids are the same as those shown in Fig. 1. Venom —1 = *Crotalus adamanteus* venom; venom —2 = *Naja naja* venom. FA signifies fatty acid.

The work in recent years has implicated phosphatidic acids or diglycerides as intermediates in the synthesis of both phosphatides and glycerides[7-9]. If this finding is true, then it may be expected that the beta-linked and one of the alpha-linked fatty acids of triglycerides have the same distribution pattern as found in the glycerophosphatides. The fact that triglycerides contain less highly unsaturated fatty acids than the phosphatides[10] may mean therefore that the fatty acids of the triglycerides undergo reduction after they have been positioned on the molecule. On the other hand, the reverse situation may prevail, namely that the fatty acids of the phosphatides undergo dehydrogenation once they are positioned on the molecule. In this regard it is noteworthy that the desaturation of fatty acids is restricted mainly to stearic to oleic, palmitic to palmitoleic and linoleic to arachidonic conversions[11,12]. If isomerization of the fatty acids occurs either on the phosphatides or triglycerides or if transesterification occurs between the various lipids the problem then becomes quite complex. Data relative to these latter processes are, however, yet forthcoming. It is possible also that the more unsaturated diglycerides are used primarily for the synthesis of phosphatides[13,14].

Since by our studies it appears that the venom phospholipase A shows preference in removing the beta-linked fatty acid on lecithins and that these fatty acids are predominantly unsaturated, it seems, in direct contrast to the assumptions of HANAHAN[15], that the unsaturated fatty acids occur to a greater extent on the beta position of these phosphatides[*]. Furthermore, the C-16 acids (in particular palmitic acid) are located primarily on the alpha position of glycerol. This conclusion can be made at least for rat-liver lecithin but whether it can be made for lecithins from other sources remains to be elucidated. This finding would be in harmony with the recent studies of MATTSON AND LUTTON[16] who have shown that in most animal fats (except lard from pig) the unsaturated fatty acids show some selection for the beta position. These observations would also be compatible with recent work which indicates that diglycerides are common intermediates in the synthesis of both triglycerides and phosphatides[7-9].

ACKNOWLEDGEMENT

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[*] In a personal communication Dr. M. KATES of the National Research Council, Ottawa, Canada, has informed the authors that he and Dr. A. TATTRIE have confirmatory evidence that the snake venom phospholipase A preferentially liberates the β-linked fatty acids of egg lecithin and that these acids are predominantly unsaturated. This work is now in press (*J. Lipid Research*).

MIXED MONOLAYERS OF β-LACTOGLOBULIN AND PHOSPHATIDES

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(Received June 11th, 1959)

SUMMARY

Mixed monolayers of β-lactoglobulin and milk phosphatides were prepared by thoroughly mixing the gaseous films of the film constituents. From the force/area curves of these mixed monolayers the amount of interaction between protein and phosphatide at different pH values was estimated. No appreciable interaction was found at pH 1.0 and 5.9, where the film constituents carry charges of similar sign. Strong complex formation, however, occurred at pH 3.9, where the protein and the phosphatide are oppositely charged. Calculations are given of the electrostatic interaction to be expected in the mixed monolayer and it is shown that the greater part of the experimental cohesion must be ascribed to non-electrical effects.

INTRODUCTION

The interaction between proteins and phospholipids has been studied extensively at air/water and oil/water interfaces, as the survey by Fraser[1] shows. It is thought that such studies may throw some light upon the nature of lipoprotein interaction and upon the physicochemical properties of biological membranes, which are often found to consist of protein and phosphatide components.

Doty and Schulman[2] and Matalon and Schulman[3] were the first to investigate the interaction by means of the spreading technique. They injected various proteins beneath films of different lipids that were spread on a Langmuir trough. The interactions that occurred were then followed by observing either the changes in the surface pressure at constant film area or the area variations at constant pressure. Apart from electrostatic effects between the protein ions and the ionic heads of the film molecules, only weak interactions between the two substances could be detected. The interaction of protein and phospholipid is strongly influenced, however, by their ionic charges. For instance, at pH values slightly below its isoelectric point the positively charged serum albumin vigorously penetrates between the negative ionic heads of a cephalin monolayer. On the other hand, by shifting the pH of the subsolution to alkaline values nearly all of the protein that had penetrated could be ejected from the phospholipid film. The latter effect is clearly due to the electrostatic repulsion between the two negatively charged components. Schulman *et al.* carried out their experiments with phospholipid films that were kept initially at surface pressures beyond the collapse pressure of protein films (*e.g.* > 15 dynes cm^{-1}). In this way spontaneous spreading of the injected protein on the surface is prevented and the detected surface pressure changes may readily be ascribed to interactions between the protein and the lipids. It must be emphasized, however, that at such high initial pressures the phosphatide film is already rather compressed; consequently the non-polar chains of the phosphatide molecules will, for steric reasons, be partly prevented from interaction with the side-chains of the protein. The difficulty has been avoided in the experiments of ELEY AND HEDGE[4], who injected the proteins at considerably lower surface pressure of the phosphatide film (*e.g.* 2 dynes. cm^{-1}). But under such conditions spontaneous spreading of the protein will occur and it will be difficult to distinguish between spreading of the protein and proper interaction effects. Thus it is still open to question, whether there is any specific interaction between protein and lipid.

A serious objection to the injection technique is that experiments are very difficult to evaluate, as was pointed out by BULL[5]. In fact one is never sure whether the injected protein is completely adsorbed on the phosphatide film since there is a possibility that it may also dissolve in the subsolution.

In the investigation on which we report here we tried to overcome these difficulties by studying the behaviour of mixed monolayers of β-lactoglobulin and milk phosphatides.

In the absence of a suitable spreading solvent for mixtures of these two substances a modified spreading technique for the mixed monolayer had to be applied. By this technique the mixed monolayer is prepared by thoroughly mixing the gaseous monolayers of the constituents, which have been spread separately.

The amount of interaction in the mixed film can easily be estimated from a comparison of the experimental force/area curve with an ideal plot. The latter is calculated from the force/area curves of the pure components, assuming that there is no interaction between them.

MATERIALS AND METHODS

Milk phosphatides were extracted from fat-free butter serum with an alcohol–carbon tetrachloride mixture and purified as described by VAN HANDEL[7] and by KOOPS[8]. The composition of the final product was found by chemical analysis to be: lecithin 30 %, cephalin 45 % and sphingomyelin 25 %. For spreading, the phosphatides were dissolved in a mixture of 80 % petroleum ether (b.p. 60—80°) and 20 % chloroform in a concentration of 0.756 mg/ml.

β-Lactoglobulin was prepared from fresh skim milk according to the method of LARSON AND JENNESS[9]. Because the final product failed to crystallize the protein was dried from the frozen state. When subjected to moving boundary electrophoresis it appeared to be homogeneous. For spreading purposes β-lactoglobulin was dissolved in barbiturate buffer pH 6.5 and ionic strength 0.1. Its concentration was about 0.5 mg/ml and fresh solutions were made every week.

Sodium chloride, used to establish a constant ionic strength in the subsolution, was heated to glowing in a platinum beaker to free it from surface-active impurities.

Acetate buffer solutions were prepared with laboratory distilled water which had passed through an "Amberlite" mixed ion-exchange bed. At the lowest surface pressures measured (*e.g.* 0.15 dynes. cm^{-1}) no surface-active contamination from this water could be detected. At pH 1.0 the subsolution was prepared from equimolar quantities of hydrochloric acid and sodium chloride.

Force/area curves at the air/water interface were determined with a conventional Langmuir through of the type described by GORTER AND SEEDER[10]. Its dimensions were 60 × 14 × 1 cm^3. Pressure readings were accurate to about 0.15 dynes. cm^{-1}.

For spreading, surface-active material was administered to the surface by means of an "Agla" micrometer syringe mounted on the micrometer of a microscope to permit a cautious approach to the surface.

The ionic strength of the subsolution was 0.2 in all experiments. On such subsolutions it was found that the spreading of β-lactoglobulin was completed within 20 minutes.

Force/area curves were most reproducible when drops of the protein solution were gently touched at the interface[5].

No difficulty was experienced in spreading the phosphatides. Pressure readings in this case were taken after 5 minutes.

We could not find a suitable spreading solvent for mixtures of β-lactoglobulin and phosphatides. Either the phosphatides form sols in the aqueous protein solution or the protein is flocculated by the apolar solvent of the phosphatides. Such "solutions" do not permit reproducible force/area measurements for the mixed monolayers.

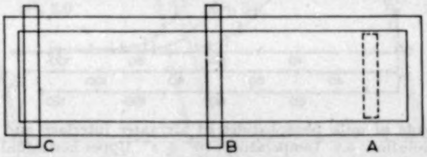

Fig. 1.

We therefore proceeded as follows: With the float of the measuring device, which is normally situated at A (Fig. 1), removed, the trough surface is divided into two equal areas by a paraffin-coated glass barrier B. Now β-lactoglobulin is spread as a gaseous film on the right-hand area. After 15 min a gaseous film of phosphatides is spread on the left compartment. After a further 5 min the barrier B is carefully removed from the surface and placed at the right-hand end of the trough. By slowly sweeping the protein film to the left it is driven into the phosphatide film. When B is again at its initial place the measuring balance is installed at A. By now moving B to the right towards the balance one can check that no surface-active material is left behind to the right of B. Then B is again removed and barrier C at the left is moved to the right to compress the mixture of the films. After several compressions and expansions the mixed monolayer is allowed to stand for a further 5 min before pressure readings are taken. The film is re-expanded and the force/area curves redetermined after different intervals of time. This check is always made and reproducibility of the surface pressures has always been good, even after three hours.

RESULTS

Force/area plots for phosphatides and β-lactoglobulin at pH 5.9, 3.9 and 1.0 are given in Figs. 2 and 3. Each of these and the following plots is the average value of

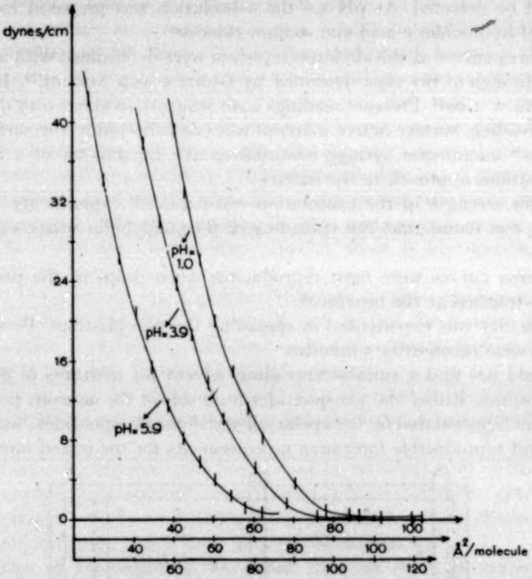

Fig. 2. Force/area plots of milk phosphatides at air/water interface and different pH values. Ionic strength of subsolution: 0.2. Temperature: 16° ± 1°. Upper horizontal axis: pH 1.0. Middle horizontal axis: pH 3.9. Lower horizontal axis: pH 5.9.

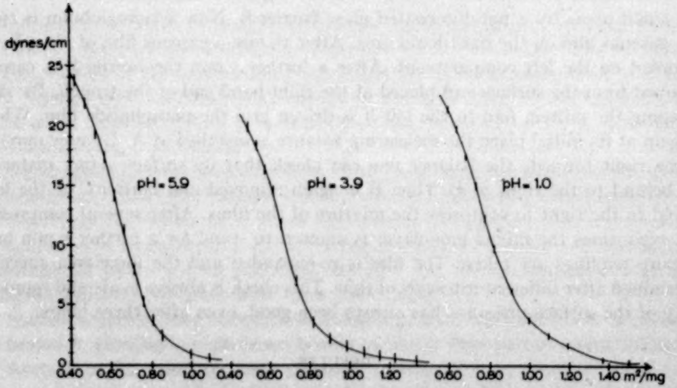

Fig. 3. Force/area plots of β-lactoglobulin at air/water interface and different pH values. Ionic strength of subsolution 0.2. Temperature: 16° ± 1°.

at least five independent measurements. For the calculation of molecular film areas in the phosphatide films of Fig. 2 an average molecular weight of 760 was used. The force/area curves of the phosphatides closely resemble those of TURNER AND WATSON[11]. They are practivally independent of the pH of the subsolution. This may be due to the zwitterion-nature of the ionic heads of lecithin and sphingomyelin and to the high value of the ionic strength of the subsolution, which tends to suppress ionization effects in the film pressure[12].

The high ionic strength also explains why the force/area curves of β-lactoglobulin at pH 5.9 and 3.9 coincide. At pH 1.0, however, the protein monolayer is highly expanded. This effect, as pointed out by CHEESMAN AND DAVIES[6] is due to the combined influence of the breaking of hydrogen bonds between the peptide chains of the film and extreme ionization of the protein at this pH. At surface pressures beyond 20 dynes. cm^{-1} the β-lactoglobulin films become unstable, and irreversible decreases in the surface pressure are observed. At pH 1.0 the protein film is considerably more reproducible than at pH 3.9 and 5.9. Obviously, denaturation by the acid subsolution enhances the protein's ability to spread.

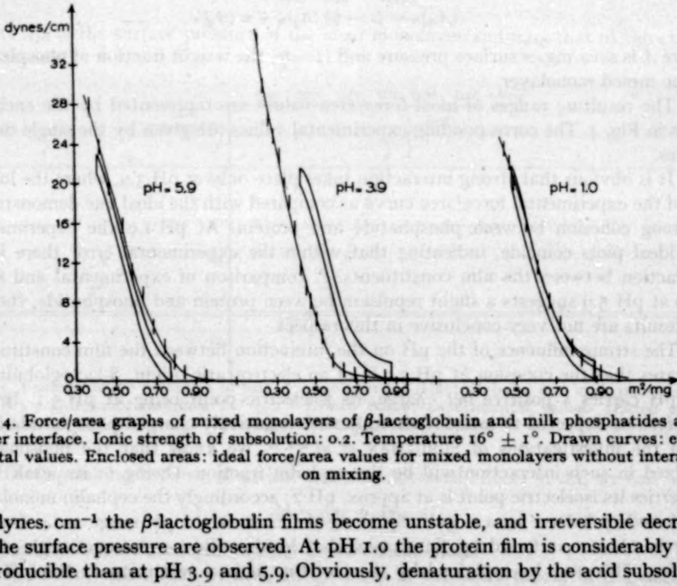

Fig. 4. Force/area graphs of mixed monolayers of β-lactoglobulin and milk phosphatides at air/water interface. Ionic strength of subsolution: 0.2. Temperature 16° ± 1°. Drawn curves: experimental values. Enclosed areas: ideal force/area values for mixed monolayers without interaction on mixing.

In Fig. 4 the drawn curves represent the force/area curves of mixed monolayers of β-lactoglobulin and phosphatides at the same pH values of 1.0, 3.9 and 5.9. The results at pH 5.9 and 3.9 were obtained by mixing 43 and 57 wt. % of protein and phosphatide, respectively. At pH 1.0 these figures were 40.3 and 59.7 %. At pH 1.0 and pH 5.9 the stability of the mixed monolayer markedly decreases when the surface pressure exceeds the collapse pressure of the β-lactoglobulin film (*e.g.* > 20 dynes. cm^{-1}), although the instability does not rise as rapidly as in the case of the pure protein monolayer. At pH 3.9 the mixed monolayer is much more stable and the film can be compressed to considerably higher surface pressures. This is due, as will be explained in the next section, to the formation of a complex between the oppositely charged protein and phosphatide constituents of the film. The positively charged β-lactoglobulin is prevented from dissolving in the subphase by the negative cephaline ions.

DISCUSSION

For an estimation of the interactions occurring in the mixed monolayers the experimental force/area curves have been compared with ideal ones in which it is assumed that there is no interaction between phosphatide and protein. The ideal force/area curves were constructed on the assumption that the specific areas of the components in the mixed monolayer would be equal to those of the pure component monolayers at constant value of the surface pressure. For the specific area in the ideal mixed monolayer we may thus write:

$$(A_m)_\pi = (1 - w)\,(A_1)_\pi + w\,(A_2)_\pi\,, \tag{1}$$

where A is area/mg, π surface pressure and $(1-w)$ the weight fraction of phosphatide in the mixed monolayer.

The resulting ranges of ideal force/area values are represented by the enclosed areas in Fig. 4. The corresponding experimental values are given by the single drawn curves.

It is obvious that strong interaction takes place only at pH 3.9, where the lowering of the experimental force/area curve as compared with the ideal one demonstrates a strong cohesion between phosphatide and protein. At pH 1.0 the experimental and ideal plots coincide, indicating that within the experimental error there is no interaction between the film constituents. A comparison of experimental and ideal plots at pH 5.9 suggests a slight repulsion between protein and phosphatide, though the results are not very conclusive in this respect.

The strong influence of the pH on the interaction between the film constituents indicates that the cohesion at pH 3.9 is of an electrostatic origin. β-lactoglobulin at this pH carries a positive net charge, its isoelectric point being at pH 5.1. It can therefore be expected to interact strongly with negatively charged phosphatide ions. From the investigations of SCHULMAN *et al.*[2,3,13], we know that the phosphatide involved in such interaction will be the cephalin fraction. Owing to its weak base properties its isoelectric point is at approx. pH 2; accordingly the cephalin monolayer was found to behave as a negatively charged film over the pH range 2–14. On the other hand, it was found by SCHULMAN that lecithin did not interact with serum proteins. This may be explained by the fact that it is a zwitterion at this pH and therefore carries no net charge. Nothing is known about the behaviour of sphingomyelin in this respect, but we may expect it to behave as lecithin, since the molecule has the same phosphatidylcholin group. We therefore feel justified in assuming that, in our experiments, it is the negatively charged cephalin fraction that interacts with β-lactoglobulin at pH 3.9.

Thus far the present results parallel the penetration experiments of SCHULMAN and co-workers as regards the influence of the pH of the subsolution. There is, however, a difference in the positions of polar and apolar groups of the protein in the mixed and in the penetrated monolayer. In the latter, the protein is adsorbed under the phosphatide film and consequently its side chains, will for steric reasons, be partly prevented from interaction with the phosphatide molecules. We may thus expect that electrostatic interactions and possibly Van Der Waals' forces and hydrogen bonding will be more pronounced in the mixed monolayer than in the penetrated one.

To investigate whether the presents results can account for such non-electrostatic interactions, let us analyse more carefully the cohesion at pH 3.9. From Fig. 4 it is evident that at this pH the mixed monolayer is highly condensed, indicating a strong attraction between the negative cephalin and positive protein ions. As a result of the mutual neutralization of the protein and cephalin ions the net charge of the monolayer nearly vanishes and the resulting decrease of the surface pressure can be estimated by the double layer theory of ionized monolayers. To this end we proceed as follows.

According to Fig. 4 the experimental values of the cohesion at a given film area are given by:

$$\Delta\pi = \pi_{\text{id}} - \pi_{\text{exp}}, \tag{2}$$

where π_{id} is the surface pressure of the ideal monolayer and π_{exp} that of the experimental one.

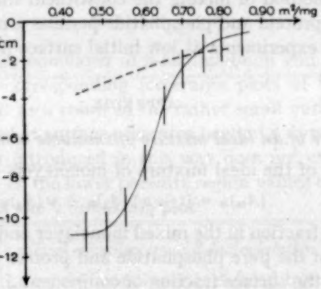

Fig. 5. Excess surface pressures of mixed monolayers of β-lactoglobulin and milk phosphatides at pH 3.9. Air/water interface 16° ± 1°. Drawn curve: experimental values. Dotted curve: charge-effect according to Gouy theory.

Distinguishing between electrical and non-electrical effects in $\Delta\pi$, we can write:

$$\Delta\pi = \Delta\pi_0 + \Delta\pi_{\text{el}}, \tag{3}$$

$\Delta\pi_0$ being the non-electrical and $\Delta\pi_{\text{el}}$ the electrical effect.

A detailed calculation of $\Delta\pi_{\text{el}}$ on the basis of the Gouy theory of the electrical double layer is given in the Appendix. The results are shown in Fig. 5, where the experimental values of $\Delta\pi$ are compared with values of $\Delta\pi_{\text{el}}$ calculated with the aid of the Gouy theory. Obviously, electrical effects in $\Delta\pi$ constitute only a minor part of it. Considering the difference between $\Delta\pi$ and $\Delta\pi_{\text{el}}$ we must bear in mind that the validity of the Gouy theory is rather restricted at the relatively high ionic strength used in this investigation. On the other hand it is known that the deviations occurring, which are due to the finite size of the counter ions and the penetration of these between the film charges, to a great extent cancel each other out[12, 15]. This confirms that the calculated values of $\Delta\pi_{\text{el}}$ are of the right order of magnitude and that the experimentally found cohesion cannot be due to electrostatic effects alone. A considerable amount of the cohesion at pH 3.9 must therefore be attributed to non-electrostatic interactions originating from complex formation between protein and phosphatide.

Of the factors that may be important in $\Delta\pi_0$ we mention the non-random distribution of the spread molecules on complex formation and the ample opportunity for short-range interactions (Van Der Waals' forces, hydrogen bonding) in the "lipoprotein" complex. Also the flexibility of the peptide chains may be decreased considerably by the neutralization of the protein charge through the adsorption of the cephalin ions[6]. All such factors tend to increase the cohesion in the mixed monolayer and should be considered when explaining the difference between $\Delta\pi$ and $\Delta\pi_{el}$.

The evidence of our experiments that non-electrostatic interactions between protein and phosphatide take place only at pH 3.9 indicates that the interpretation of penetration experiments at low initial surface pressures and neutral pH must be done with great care. At least part of the observed increases of the surface pressure in such penetration experiments may be due to the spontaneous spreading of the protein at the low initial surface pressures applied to the phosphatide film. In our opinion the present method of mixing the constituent monolayers in order to study interactions between protein and phosphatide permits a more reliable interpretation than the penetration experiments at low initial surface pressures.

APPENDIX

I. *The surface pressure of an ideal mixture of insoluble monolayers*

The specific area of the ideal mixture of monolayers is defined by:

$$(A_m)_\pi = (1 - w)(A_1)_\pi + w(A_2)_\pi , \tag{1}$$

where w is the weight fraction in the mixed monolayer and A_1 and A_2 the area per mg of spread substance in the pure phosphatide and protein monolayers.

According to (1) the surface fraction of component 2 in the mixed monolayer is:

$$y = wA_2/A_m , \tag{4}$$

and likewise the surface fraction of component 1:

$$(1 - y) = (1 - w)A_1/A_m . \tag{5}$$

Gibbs' adsorption theorem for an insoluble monolayer of 2 components reads[16]:

$$\mathrm{d}\pi_m = \Gamma_1 \,\mathrm{d}\mu_1 + \Gamma_2 \,\mathrm{d}\mu_2 , \tag{6}$$

Γ_1 and Γ_2 being the amounts of component 1 and component 2 per unit area of monolayer and μ_1 and μ_2 their respective thermodynamic potentials.

Assuming that the components 1 and 2 form an ideal mixture of constant composition we may write:

$$\mathrm{d}\mu_1 = \mathrm{d}\mu_1^0 ,$$

and

$$\mathrm{d}\mu_2 = \mathrm{d}\mu_2^0 ,$$

the superscript ^{0} denoting the pure component monolayers. Hence, applying (6) to an ideally mixed monolayer we obtain:

$$\mathrm{d}\pi_{id} = \Gamma_1 \,\mathrm{d}\mu_1^0 + \Gamma_2 \,\mathrm{d}\mu_2^0 . \tag{7}$$

For the Gibbs' equation of the pure component monolayers we have:

$$\mathrm{d}\pi_1^0 = \Gamma_1^0 \,\mathrm{d}\mu_1^0 , \tag{8}$$

and

$$\mathrm{d}\pi_2^0 = \Gamma_2^0 \,\mathrm{d}\mu_2^0 . \tag{9}$$

Substituting (8) and (9) into (7) we obtain:

$$d\pi_{1d} = (\Gamma_1/\Gamma_1^0)\, d\pi_1^0 + (\Gamma_2/\Gamma_2^0)\, d\pi_2^0 = \{(1-w)\,\Gamma_m/\Gamma_1^0\}\, d\pi_1^0 + \{w\,\Gamma_m/\Gamma_2^0\}\, d\pi_2^0,$$

where Γ_m is the total amount of spread substances per unit area of the mixed monolayer.

Since $\Gamma_m = 1/A_m$, $\Gamma_1^0 = 1/A_1$ and $\Gamma_2^0 = 1/A_2$ the latter equation reduces to:

$$d\pi_{1d} = \{(1-w)A_1/A_m\}\, d\pi_1^0 + \{wA_2/A_m\}\, d\pi_2^0 = (1-y)\, d\pi_1^0 + y\, d\pi_2^0 \tag{10}$$

Integrating (10) we obtain for the surface pressure of the ideally mixed monolayer

$$\int_0^{\pi} d\pi_{1d} = \int_0^{\pi} (1-y)\, d\pi_1^0 + \int_0^{\pi} y\, d\pi_2^0 \tag{11}$$

From (11) we see that it is possible to express π_{1d} explicitly in the surface pressure π_1^0 and π_2^0 of the pure component monolayers only if the dependence of y on surface pressure is known. For the particular case that y is constant (11) simplifies to:

$$\pi_{1d} = (1-y)\,\pi_1^0 + y\,\pi_2^0 \tag{12}$$

In view of the ideally mixed monolayer of β-lactoglobulin and milk phosphatides we may estimate y from the corresponding force/area plots of the ideal and protein monolayers. It is found that as a result of the rather small variations in y the simple equation (12) may be applied at surface pressures beyond 5 dynes. cm^{-1}, using a mean value $y_m = 0.51$. The error introduced in this way does not exceed 5 % of the total area of the integrals in (11). In the lower pressure region values of y_m can be calculated by graphical integrations of the y *versus* π_{1d} plot.

II. *Electrostatic interaction in mixed monolayers of β-lactoglobulin and milk phosphatides*

The cohesive force in the mixed monolayer is defined by:

$$\Delta\pi = \pi_{1d} - \pi_{exp} \tag{2}$$

The surface pressure of ionized monolayers quite generally is given by:

$$\pi = \pi_0 + \pi_{el}$$

π_0 being the surface pressure of the unionized monolayer and π_{el} the pressure increment due to its ionization. Consequently, we may write for $\Delta\pi$:

$$\Delta\pi = \Delta\pi_0 + \Delta\pi_{el} \tag{3}$$

From general thermodynamic considerations it was deduced (12) that

$$\pi_{el} = \int_0^{\psi} \sigma\, d\psi,$$

in which σ is the monolayer charge per cm^2 and ψ the corresponding monolayer potential.

From the expression (12) for π_{1d} deduced in the preceding section $\Delta\pi_{el}$ thus becomes:

$$\Delta\pi_{el} = \left[(1-y_m)\left\{\int_0^{\psi_1} \sigma_1\, d\psi_1\right\}_{A_1} + y_m\left\{\int_0^{\psi_2} \sigma_2\, d\psi_2\right\}_{A_2}\right] - \left\{\int_0^{\psi_{exp}} \sigma_{exp}\, d\psi_{exp}\right\}_{A_m}. \tag{13}$$

The monolayer charges in equation (13) are given by:

$$\sigma_1 = -\Gamma_1 e$$

$$\sigma_2 = +\Gamma_2 v e$$

$$\sigma_{\mathrm{exp}} = (\Gamma_2 v - \Gamma_1) e$$

where Γ_1 is the number of cephalin ions per cm^2 of monolayer and Γ_2 the corresponding number of protein ions at the film areas indicated in (13); e is the absolute value of the electronic charge, and v the valency of the protein at pH 3.9.

For an estimation of Γ_2 and v we need to know the molecular weight of β-lactoglobulin and its charge at pH 3.9. For the former we accept a value of 40,000[5,14]; the charge can be estimated from the titration curve to be $+ 22\ e$ at pH 3.9 and ionic strength 0.2[16].

According to the Gouy theory (12):

$$\int_0^{\psi} \sigma\, \mathrm{d}\,\psi = 6.1\, c^{1/2}[\cosh\{\text{arc sinh}\,(28.10^{-6}\,\sigma\, c^{-1/2})\} - 1], \tag{14}$$

where c is the equivalent concentration of electrolyte in the subsolution and σ the monolayer charge expressed in electrostatic units per cm^2.

Application of eqns. (13) and (14) to the present experiments at pH 3.9. leads to the values of $\Delta\pi_{\mathrm{el}}$ represented by the dotted curve of Fig. 5.

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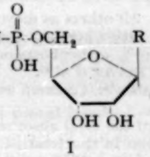
Short Communications

Synthesis of ribonucleoside-5′-polyphosphates

The preparation of ribonucleoside-5′-phosphoramidates (I) by a simple, one-step procedure[1] has provided the key intermediates for a new synthetic approach to unsymmetrical nucleoside-5′-pyrophosphates of biological interest such as ADP[2], UDP[3], FAD[4] and UDPG[4].



I

R = adenine, guanine, cytosine or uracil

All of the reactions which have been studied so far involve coupling a phosphoramidate with orthophosphoric acid or a phosphomonoester. This report describes the reaction of nucleoside-5′-phosphoramidates with pyrophosphoric acid, to give nucleoside-5′-triphosphates.

Some selected data from studies on the reaction

$$\mathrm{AMP\text{-}NH_2 + PP \rightarrow ATP + NH_3}$$

are shown in Table I. The reactions were carried out in 12-ml centrifuge tubes under anhydrous conditions at 0° with rapid stirring. The products were precipitated with 1.4 ml petroleum ether (b.p. 30–60°), collected by centrifugation, washed twice with 1-ml portions of anhydrous ether and once with acetone. The residue was dissolved in 1.4 ml 1 *N* NH_4OH and fractionated by paper electrophoresis (Whatman 3 MM paper, saturated with 0.02 *M* citrate buffer, pH 4, 29 V/cm). The u.v.-absorbing spots were eluted from the paper and the absorbance was determined in a Beckman Model DU spectrophotometer. The presence of unreacted amide was detected by paper chromatography in isopropanol–ammonia–water (7 : 1 : 3)[5].

The results show that after 40 min considerable unchanged AMP–NH_2 was still present (Expt. 1, monophosphate fraction). After 90 min (Expt. 2), the reaction was complete and increasing the reaction time had very little effect on the product distribution. Increasing the ratio of PP : AMP–NH_2 (Expt. 3) gave a slight improvement in the ADP–ATP yield, apparently by diminishing the formation of DAPP.

Abbreviations: AMP, adenosine-5′-monophosphate; AMP–NH_2, adenosine-5′-phosphoramidate; ADP, adenosine-5′-diphosphate; DAPP, P^1P^2-diadenosine-5′-pyrophosphate; ATP, adenosine-5′-triphosphate; CMP–NH_2, cytidine-5′-phosphoramidate; GMP–NH_2, guanosine-5′-phosphoramidate; UMP–NH_2, uridine-5′-phosphoramidate; UDPG, uridine diphosphate glucose; FAD, flavin-adenine dinucleotide; P_i, orthophosphoric acid; PP, pyrophosphoric acid.

TABLE I

REACTION OF NUCLEOSIDE-5-PHOSPHORAMIDATES WITH PYROPHOSPHORIC ACID

				Solvent		Products			
Expt.	*Amide*[*]	*PP/amide*[**]	*Time*	*o-chlorophenol*[***]	*n-butanol*	*Monophosphate*	*Diphosphate*	*Triphosphate*	*Symm. pyrophosphate*
		mg/mg	*min*	*ml*	*ml*	%[§]	%	%	%
1	AMP–NH$_2$	2	40	0.7	0	66[§§]	16	9	9
2	AMP–NH$_2$	2	90	0.7	0	31	35	24	10
3	AMP–NH$_2$	5	90	0.7	0	32	40	28	0
4	AMP–NH$_2$	7	180	0.2	0.5	54	11	30	5
5	CMP–NH$_2$	2	180	0.2	0.5	37	18	24	21[§§§]
6	GMP–NH$_2$	3	210	0.2	0.5	46	10	32	12
7	UMP–NH$_2$	2	120	0.7	0	52	16	19	13

[*] GMP–NH$_2$ as its ammonium salt. All others as dicyclohexylguanidinium salts.

[**] Crystalline pyrophosphoric acid, City Chemical Corp., New York. This sample contained about 60% pyrophosphoric acid and 40% orthophosphoric acid identified by paper chromatography[11] and assayed by the method of FLYNN *et al.*[12].

[***] Purified from practical grade (Matheson, Coleman and Bell, New York) by fractional distillation through a 4-foot packed column.

[§] Results are expressed as percent of the total absorbance of all spots.

[§§] Considerable AMP–NH$_2$ was present in this fraction.

[§§§] This spot was not definitely identified as P^{1}P^2-dicytidine pyrophosphate.

Thus, under the most favorable conditions found so far, a total yield of ADP + ATP of 68 % has been obtained (Expt. 3).

Several solvents other than *o*-chlorophenol were also investigated. However, no single solvent was found which would dissolve the reactants and therefore the reaction was very slow. For example, when *n*-butanol was used, only traces of ADP and ATP could be detected after 3 days and large amounts of unreacted AMP–NH$_2$ were present. However, when a mixture of *n*-butanol and *o*-chlorophenol was employed, the reaction was complete in 180 min (Expt. 4). It is most interesting that this solvent mixture gave a product distribution completely different from *o*-chlorophenol alone. This change in ADP : ATP ratio from 1.4 (*o*-chlorophenol, Expt. 3) to 0.3 (*o*-chlorophenol + *n*-butanol, Expt. 4) was reproducible in 4 different experiments.

The reaction of other nucleoside-5′-phosphoramidates with pyrophosphoric acid has not been investigated as extensively as the AMP–NH$_2$ reaction. However, moderate yields of the corresponding di- and triphosphates have been obtained as shown in Table I (Expts. 5, 6 and 7).

No concerted effort has been made to isolate the products from these small-scale reactions. However, ion-exchange separation of the products expected from the AMP–NH$_2$: PP reaction was investigated. The order of elution from a Dowex-1-chloride column (1 × 3 cm, 200–400 mesh, 8 % cross-linked) using linear gradient elution[6,7] (500 ml 0.003 *N* HCl in the mixer, 500 ml 0.003 *N* HCl + 0.1 *M* LiCl in the reservoir) was AMP, P$_i$, ADP, PP, DAPP and ATP. By this procedure, ADP and ATP can be separated easily from the other reaction products and similar separations should be possible with other nucleotides[8].

In attempting to formulate a mechanism to explain the product distribution, two points need to be emphasized. First of all, numerous experiments in this laboratory have established that the amides are extremely sensitive to moisture in acidic solutions and in small-scale experiments such as those described here, it is virtually impossible to eliminate traces of water from the reaction mixtures. Thus, at least part of the AMP is probably produced by hydrolysis of AMP–NH_2. Further reaction of AMP with AMP–NH_2 could then account for the small amount of DAPP which is usually found. Secondly, samples of crystalline commercial pyrophosphoric acid employed in this work contained as much as 40 % orthophosphoric acid which could react directly with AMP-NH_2 to produce ADP. However, the change in ADP : ATP ratios and particularly the large amount of AMP formed when the solvent is changed is difficult to reconcile with this simple explanation. It is possible that exchange reactions of the type

$$2ADP \rightleftharpoons AMP + ATP \qquad (1)$$

$$ATP + Pi \rightleftharpoons ADP + PP \qquad (2)$$

also occur and influence the product distribution. If reaction (1) occurs, the solvent appears to play an important role since no ATP could be detected when AMP–NH_2 reacted with P_i in *o*-chlorophenol to give ADP[2]. Reaction (2) has been demonstrated by LOWENSTEIN in the presence of metal ions[9].

Recently, the biological counterpart of the work described here has been reported by ELLFOLK AND KATUNUMA[10]. These workers described crude enzyme preparations from several bacteria which catalyze the reaction

$$ATP + NH_3 \rightleftharpoons AMP\text{–}NH_2 + PP$$

Thus, the nucleoside-5′-phosphoramidates assume new importance as biological intermediates. It will be interesting to see what actual role these compounds play in metabolic reactions and if nucleoside phosphoramidates other than AMP–NH_2 have any biological activity.

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A ribonuclease from *Nepenthes* spp

Insectivorus plants have a variety of specialised mechanisms for capturing insects and, presumably, for using the products of digestion or autolysis of the insect bodies. Some 400 species of insectivorus plants have been recorded, but little attention appears to have been paid to the enzymes which may be present in the secretions which they produce. About two years ago we examined the secretions from several species of *Nepenthes* and found they contained substantial ribonuclease activity, with a specificity differing from pancreatic ribonuclease. Since the mode of action of the *Nepenthes* ribonuclease appears to be similar to others recently described from pea leaves[1,2], tobacco leaves[3,4] and ryegrass[5], a condensed account of our results is given here to provide information on an enzyme that is relatively simple to prepare for use.

We have tested four sources of the secretion: from *Nepenthes gracilis*, *N. ampullaria* and *N. rafflesiana* collected in a forest near Singapore, and from an unidentified, large-pitchered species growing in the Auckland Botanic Gardens. The liquid from the pitcher is filtered to remove debris, mainly remains of insect bodies. It may then contain 0.1–1.0 mg/ml of total solids and has a pH of about 3. RNA from *Bacillus cereus* or from TMV was used as substrate and the methods for determination of products were largely those developed by MARKHAM AND SMITH and summarised by MARKHAM[6]. Ribonuclease activity was maintained over the pH range tested (2–9) but was greatest at pH 5. Tests were routinely made with secretion brought to pH 5 with acetate buffer (0.1 M final concentration). After a few-hours' incubation at pH 5 and 37° the secretion degrades TMV RNA almost quantitatively to the four nucleoside 2′,3′-cyclic phosphates. The pyrimidine compounds are resistant to further action, but the purine cyclic phosphates are cleaved at a slower rate to the 3′-monophosphates. Adenosine 2′,3′-cyclic phosphate is attacked at abouttwice the rate of the guanine compound. In the first sample of the secretion we tested (from the Auckland source) phosphatase activity was not detected. If present, it had less than 0.1 % the rate of the nuclease activity. In all the other samples tested (including material from plants grown from cuttings from the first source) variable amounts of phosphatase activity were found.

Ribonuclease activity is slowly lost over a period of months on storage of the secretion at 4°, and more slowly when stored frozen. There is no detectable loss of activity on heating at pH 5 to 70° for 5 min but over 95 % activity is lost on heating to 80° for 5 min. The activity for the conversion of adenosine 2′,3′-cyclic phosphate to the 3′-phosphate shows a parallel sensitivity to heat.

The mode of action of *Nepenthes* ribonuclease appears to be closest to that described from tobacco leaves[1,5].

The function of the nucleases found in leaves is unknown. In view of its occurrence in the pitcher secretion the *Nepenthes* enzyme very probably has a strictly degradative function, making constituents of the RNA from insect bodies available to the plant. Incubation of *Nepenthes* secretion under a range of conditions with the various 2′,3′-cyclic phosphates gave no indication that the enzyme has any ability to cause polymerization of the nucleotides, but it would be preferable to make such tests with a purified preparation of the enzyme.

Abbreviations: RNA, ribonucleic acid; TMV, tobacco mosaic virus.

My sincere thanks are due to Mr. H. M. BURKILL, The Botanic Gardens, Singapore 10, Malaya, for supplying samples of *Nepenthes* secretion. This work was supported in part by a grant from the Rockefeller Foundation.

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The reactivation of the succinate-cytochrome *c* reductase system by a hydrocarbon residue

There are many reports[1–12] on the reactivation of isooctane-extracted cytochrome preparations by fat-soluble vitamins and a variety of lipids. From the present communication it should be recognized that following the evaporation or redistillation of practical or spectral-grade isooctane (2,2,4-trimethylpentane, No. P2396 and No. S2396, Eastman Organic Chemicals, Distillation Products Industries and Phillips Petroleum Company) a lipid-like residue has been found which is fully able to reactivate isooctane-extracted succinate-cytochrome *c* reductase preparations. Column- and paper-chromatographic analysis, chemical tests and spectral analysis demonstrate that this active residue is a non-polar, branched hydrocarbon.

In the course of the determination of the succinate-cytochrome *c* reductase reactivating ability of various lipid fractions obtained by silicic acid column chromatography[13] of the isooctane-extracted lipids from particulate cytochrome preparations, it was found that the most marked reactivating activity occurred in a lipid fraction (Fraction C, Table I) which was eluted by petroleum ether and which constituted less than 1 % of the total lipid. This lipid fraction was observed on paper chromatograms (carried out on paper impregnated with silicic acid, using as solvent *n*-heptane–diisobutyl ketone (96:6)[14]) of the isooctane-extracted lipids of the cytochrome preparations as a rapid-moving spot with an R_F value of 0.85. The spot appeared yellow when viewed under u.v. light following staining with rhodamine-G. However, this lipid fraction was not present when the particulate cytochrome preparations were extracted by the conventional chloroform–methanol method[15] rather than with isooctane. An examination of the active lipid material obtained by isooctane extraction of the cytochrome preparations showed that this material was initially present in the practical-grade isooctane which was used to extract the enzyme system and that it was also present to a lesser extent in spectral grade and redistilled practical grade isooctane.

A sample of the material present in the isooctane was obtained by the distillation of 4000 g of practical-grade isooctane. The resulting yellow oily residue (41 mg) was

TABLE I

REACTIVATION OF ISOOCTANE-EXTRACTED SUCCINATE–CYTOCHROME *c* REDUCTASE PREPARATIONS

*System**	*Per cent restoration of succinate-cytochrome c reductase activity***
Non-extracted preparation – control	100
Extracted preparation	18
Extracted preparation + (A)	46
Extracted preparation + (B)	36
Extracted preparation + (C)	108
Extracted preparation + (D)	95

* (A) 100 μg α-D-tocopherol.

(B) 100 μg of total lipid obtained by isooctane extraction of a cytochrome *c* reductase preparation.

(C) 100 μg of a fraction obtained by silicic acid column chromatography of the isooctane lipid extract (lipid B) of a cytochrome *c* reductase preparation. Fraction (C) is eluted from silicic acid by petroleum ether.

(D) 100 μg of a fraction obtained by silicic acid column chromatography of the residue remaining after distillation of isooctane alone. Fraction (D) is eluted from silicic acid with petroleum ether.

** The preparation, extraction, and assay of the enzyme system were performed as previously described[7,8].

dissolved in 5 ml petroleum ether and placed on a 10-g silicic acid column and then eluted with 20 ml petroleum ether. Following solvent evaporation a colorless oily residue (32 mg) was obtained which was able fully to reactivate an isooctane-extracted succinate-cytochrome *c* reductase preparation (Fraction D, Table I). Paper-chromatographic analysis, chemical tests, and the u.v. spectrum gave strong evidence for the identity of the isooctane residue with the active lipid fraction obtained by column fractionation of isooctane extracts of particulate cytochrome preparations. This material was free from P and N, gave negative tests with ninhydrin and with reagents for the detection of ketones, aldehydes, sugars, esters, unsaturation, choline and cholesterol[13,15,16]. The infrared spectrum* of the active material was in agreement with that of a branched, saturated, non-polar, long-chain hydrocarbon. The major absorption bands were at 3.40 μ, 3.48 μ, 6.83 μ, 7.25 μ and 13.85 μ. The absence of absorption bands characteristic of reactive functional groups was in agreement with the findings of the chemical tests. The u.v. spectrum of this material revealed a broad peak at 250 mμ. A dimerization product of isooctane fits the available evidence.

The above findings are in accord with the suggestion that a long hydrocarbon chain is a common feature of and may be responsible for the ability of various substances to reactivate an extracted cytochrome *c* reductase preparation[8,11,12]. The branched isoprenoid-like character of the hydrocarbon may be of particular interest since the structural unit occurs in several biologically active compounds. The above findings also suggest that caution must be exercised in evaluating the activity of any lipid residue which is obtained by isooctane extraction.

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Synthesis of amino acids from carboxylic acids by isolated rat diaphragm

Manchester and Krahl[1] have recently shown that isolated rat diaphragm will incorporate ^{14}C from a variety of carboxylic acids into its protein. This communication describes the results of experiments in which, by acid hydrolysis of such protein samples and separation of amino acids by column chromatography, the location and nature of the ^{14}C incorporated into diaphragm protein from the various carboxylic acids has been determined.

The samples of protein had been prepared in experiments reported elsewhere[1]. The method for the hydrolysis and separation of amino acids was as described by Manchester and Young[2], except that the 1.5 *N* HCl used for elution was replaced by 1 *N* HCl. The results are shown in Table I.

No significant radioactivity was found in any fraction other than those described below. ^{14}C from [1,5-$^{14}C_2$]citrate and [1-^{14}C]acetate was found largely in the glutamic acid fraction and to a less extent in the aspartic acid fraction. ^{14}C from [2-^{14}C]succinate, [1-^{14}C]isobutyrate and [1-^{14}C]propionate was found mainly in the aspartic acid and glutamic acid fractions to a roughly equal extent, but a small portion was also detected

TABLE I

LOCATION AND DISTRIBUTION OF ^{14}C IN PROTEIN OBTAINED FROM RAT DIAPHRAGM WHICH HAD BEEN INCUBATED *in vitro* WITH ^{14}C-LABELLED CARBOXYLIC ACIDS

	Radioactivity in hydrolysate from 10 mg protein					
		Percentage of ^{14}C found in				
Diaphragm incubated with	*Total counts/min eluted from column*	*Aspartate*	*Serine + threonine*	*Glutamate*	*Alanine*	*Other amino acids*
[1,5-^{14}C$_2$]citrate	214	34	0	66	0	0
[2-^{14}C]succinate	1139	44	0	49	7	0
[1-^{14}C]isobutyrate	187	40	0	47	13	0
[1-^{14}C]propionate	6981	51	0	41	8	0
[1-^{14}C]acetate	5570	24	0	76	0	0
[^{14}C]formate	319	0	100	0	0	0

in the alanine fraction. ^{14}C from [^{14}C]formate was found only in the fraction containing serine plus threonine.

The incorporation of ^{14}C from formate into serine would be consistent with the formation of an active one-carbon fragment[3] from formate in diaphragm and the synthesis of serine from glycine by addition of an active one-carbon fragment, as had been previously found[2,4]. Both propionate[5] and isobutyrate[6] are believed to be broken down in the animal body to succinate. That the distribution of ^{14}C incorporated into protein derived from these three substrates is similar is compatible with this view. Aspartic acid is most probably formed from succinate by operation of the tricarboxylic acid cycle and subsequent amination of oxaloacetate so formed. The mechanism whereby ^{14}C from succinate enters glutamic acid is not known with certainty, but might be the reverse of the ketoglutarate dehydrogenase reaction in which CO_2 is fixed with succinyl-CoA or succinic semi-aldehyde to form ketoglutarate, which can then be aminated to glutamic acid[2]. As is to be expected, entrance of labelled intermediates of the tricarboxylic acid cycle between ketoglutarate and oxaloacetate leads to a different pattern of labelling of the glutamic and aspartic acids from that which is observed when diaphragm is incubated with acetate or citrate.

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Some properties of a soluble and particle-bound hydrogenase in *Aerobacter aerogenes*

It is well established that, with the exception of the *Clostridium* type, hydrogenase in homogenates of bacteria is localized in the particles. Many attempts have been made to bring the particle-bound hydrogenase into solution but only in one case has this been successful[1]. In this paper, experiments are reported in which hydrogenase from *Aerobacter aerogenes* was obtained either in soluble or particle-bound form by varying the procedure of cell breakage.

Cells of *A. aerogenes* were grown in mass culture as already described by PENGRA AND WILSON[2]. Preparations in which hydrogenase was associated with the particles were obtained by disrupting the cells with a Hughes press and separating the homogenate by differential centrifugation into the following fractions which were designated according to the nomenclature of ALEXANDER AND WILSON[3]: 0.1 s 20 (crude extract), 9.7 p 30, 9.7 s 30, 144 p 60 and 144 s 60. Soluble hydrogenase was prepared by breaking the cells for 10 min in the Raytheon magnetostrictive sonic oscillator. Whole cells and large cell debris were removed by centrifugation at 25,000 × *g* for 10 min. The supernatant (25 s 10) was centrifuged into the fractions 144 s 60 and 144 p 60.

For the particle-bound hydrogenase, measurement of H_2 uptake in the presence of benzylviologen and H_2 evolution from reduced methylviologen were the most suitable assays. Although when assayed with methylene blue as acceptor the enzyme activity of the soluble preparations was about 3 times greater than the activity measured by the evolution assay, the results with methylene blue were variable. Soluble hydrogenase preparations assayed by H_2 uptake in the presence of methyl- or benzylviologen showed about the same activity as with the evolution assay. For comparison, therefore, the evolution assay was used throughout for both preparations.

Data in Table I demonstrate that the hydrogenase activity of the Hughes press preparation is mainly localized in the particle fraction (9.7 p 30) while the activity of the sonic-treated preparation is still entirely in the supernatant fraction (144 s 60).

The stability of these two preparations was also quite different. The particle-bound hydrogenase (fraction 9.7 p 30) lost activity in 2 h when stored in air at 4°

TABLE I

HYDROGENASE ACTIVITY OF THE PARTICLE-BOUND AND SOLUBLE HYDROGENASE FROM *Aerobacter aerogenes*

Activity measured at 30° with 0.05 *M* phosphate, pH 7, and 40 µmoles reduced methylviologen in a reaction vol. of 3 ml. Gas phase, helium.

Method of cell disruption	*Fraction*	*Activity ($\mu l\ H_2/h/mg\ N$)*
Hughes press	0.1 s 20	2,300
	9.7 p 30	7,200
	9.7 s 30	530
	144 p 60	610
	144 s 60	0
Sonic treatment	25 s 10	13,300
	144 p 60	0
	144 s 60	11,200

and had a half life of about 2 days when stored under H_2 at 4°. Although fraction 25 s 10 of the sonic extract also lost activity when stored under air at 4° for 24 h, it was stable for 5 days when kept under H_2 at 4°.

The action of some inhibitors is shown in Table II. Although we failed to demonstrate light reversibility of CO inhibition of hydrogenase activity the experiments with cyanide, *o*-phenanthroline and ethylenediaminetetraacetate suggest that a metal, probably iron, is involved in the hydrogenase system.

TABLE II

EFFECT OF SOME INHIBITORS ON AEROBACTER HYDROGENASE ACTIVITY

Conditions as in Table I.

Inhibitor	*Concentration*	*Inhibition (%)*	
		Sonic extract (fraction 25 s 10)	*Hughes-press preparation (fraction 9.7 p 30)*
CO	10%	43.0	30.0
	20%	67.0	85.0
	30%	82.0	95.0
KCN	$3.3 \cdot 10^{-4}$ *M**	30.0	—
	$3.3 \cdot 10^{-3}$ *M**	95.0	80.0
	$33.0 \cdot 10^{-3}$ *M**	100.0	100.0
	$3.3 \cdot 10^{-3}$ *M** + 100 µmoles Fe^{2+}	34.0	—
	$3.3 \cdot 10^{-3}$ *M***	10–40	—
o-phenanthroline	$16.6 \cdot 10^{-3}$ *M*	51.0	—
	$33.3 \cdot 10^{-3}$ *M*	69.0	50.0
Ethylenediamine tetraacetate	$66.6 \cdot 10^{-3}$ *M*	73.5	75.0
	$> 66.6 \cdot 10^{-3}$ *M*	100.0	—

* Preincubated with cyanide.
** No preincubation.

No H_2 was liberated from pyruvate by any preparation, but CO_2 was evolved and acetoin was formed at an appreciable rate by the sonic extract (25 s 10). With the Hughes-press preparation only fraction 144 s 60 carried out these reactions.

All attempts to bring the particle-bound hydrogenase (fraction 9.7 p 30) into solution by sonic treatment caused a total loss of activity.

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Improved protein resolution of concentrated tissue extract in zonal column electrophoresis

Zonal electrophoresis in vertical columns of ethanolyzed cellulose[1] has been developed by Porath[2–5] into a versatile and highly useful preparative and analytical procedure.

Investigations on the soluble proteins of rat liver[6,7] have extended into column electrophoretic studies, begun by one of us at the Institute of Biochemistry, Uppsala University, Sweden. Isolation and analysis of all principal components therein[6,7] require use of extracts of high protein concentrations and viscosities. Introduction of such samples 15 to 25 cm into the column, in allowance for electroosmosis, results in broad and irregular starting zones and consequently inferior protein resolution. Porath[3] devised a sectional column permitting introduction of viscous samples into the middle of a column. This provided only preliminary fractionation for further efficient electrophoretic resolution. This communication deals with a convenient modification of the Porath technique. In single-stage runs, thus reducing alterations attending dilution and concentration of labile proteins, it provides improvement in resolution of concentrated extracts and certain technical advantages.

Two pools of soluble proteins of livers of rats fed the hepatocarcinogen, 3′-methyl-4-dimethylaminoazobenzene for 2.5–3 weeks were prepared[7,8]. Each was divided into equal portions, dialyzed against 0.02 I sodium veronal + 0.03 I NaCl, pH 8.6, and made to a concentration close to 10.0 % protein. 7.5 ml were introduced at 6–25 ml/h into a Porath column of ethanolyzed cellulose, 225 × 3.4 cm, water-cooled peripherally at 1–2° and previously equilibrated with the same buffer (Fig. 1).

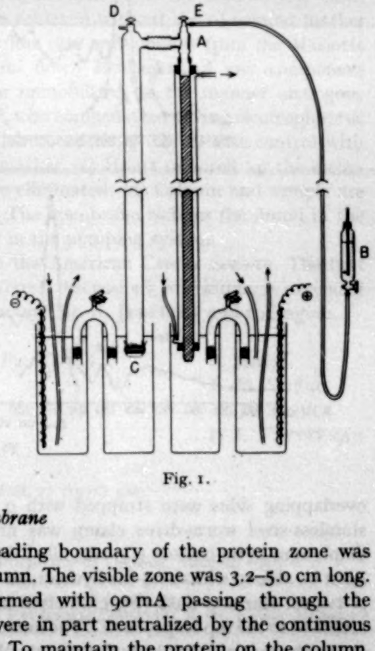

Fig. 1.

Electrophoresis without semi-permeable membrane

To allow for electroosmosis[2,9], the leading boundary of the protein zone was introduced 10.4 cm (minimal) into the column. The visible zone was 3.2–5.0 cm long. Subsequently, electrophoresis was performed with 90 mA passing through the cellulose circuit. Products of electrolysis were in part neutralized by the continuous peristaltic circulation of electrode buffers. To maintain the protein on the column, despite electroosmotic back-movement, the current was stopped at 20, 40 and 60 h, (A) closed off, and protein was compensated down 12 cm, 12 cm, and 4 cm, respectively, as measured by liquid flow at 22–25 ml/h from a graduated 125 ml Mariotte flask (B). After a total of 80 h of electrophoresis, protein was eluted at 30 ml/h and collected in 10.0-ml fractions at 7°. Protein was measured in the Beckman DU Spectrophotometer at 284 mμ[10] against veronal chloride buffer. For the evaluation of zone sharpness and resolution, distribution of the dye content of azo protein therein[7, 11] was analyzed by the formic acid method of WIRTZ, MILLER AND MILLER[11].

Electrophoresis with membrane-closed column

Similarly, the leading protein boundary was introduced into the same column only 2.5 cm. The visible zone was 1.6 cm long. Two layers of Visking dialysis membrane of regenerated cellulose were stretched over the bottom of the vertical liquid arm; overlapping sides were strapped with 0.75 in. rubber splicing tape, over which a stainless-steel worm-drive clamp was firmly fastened. The liquid tube, with the diaphragm at its bottom (C), was filled through the top opening (D). (The membrane must be able to withstand the hydrostatic pressure of the liquid arm without leaking.) A 125-ml Mariotte flask (B), graduated in 1-ml divisions and containing buffer, was attached to the top stopcock (E). The immersed capillary opening was positioned 10 cm above the meniscus of the buffer vessels. With this flask just previously opened to the liquid above the column, the column and vertical liquid arm were joined through glass joint (A). Throughout the ensuing electrophoresis, the flask was positioned for a flow of 2.5 to 3.5 ml/h. Elution and analysis followed as above.

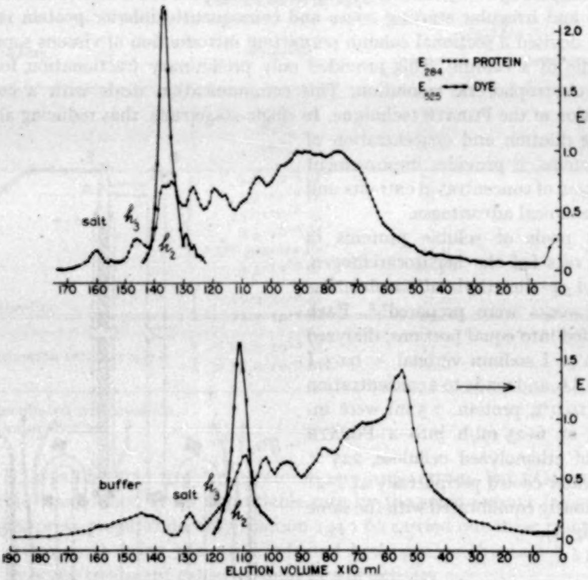

Fig. 2.

Fig. 2 compares the protein and protein-azo dye separations obtained. Similar results were realized with both protein pools. Improved resolution in the protein and azoprotein distributions with the membrane-closed column is evident from the sharpness, height, detail and additional peaks obtained (upper pattern). Thus, the h_3 component is considerably better resolved using the present technique than without membrane (lower pattern). The h_2 and other components divide into at least two subcomponents, unlike the latter case. The peak dye content of h_2 azoproteins is considerably taller ($E_{525\,m\mu} = 2.20$) than in the matching run ($E_{525\,m\mu} = 1.63$). Faster minor azoprotein peaks are evident in the former, whereas they merge into an almost smooth leading shoulder in the latter case. With this improved resolution, the peak content of azoproteins has been determined to coincide exactly with the slow subdivision of the h_2 component, the slow h_2 component[7]. Studies[8] utilizing the membrane-closed column are in progress.

The membrane-closed column has additional advantages. (a) Electroosmotic backflow of buffer is greatly reduced. Compared to open-column electrophoresis with discontinuous forward compensations, the sample in the membrane-closed column travels less distance and spreads less therefrom, and is in the column less time. (b) Precise control of compensation may be achieved without use of colored marker substance by the adjustment of the liquid flow rate and amount from the Mariotte flask. (A pump could automatically control flow.) Movement of any component may be thus more accurately controlled or immobilized, in the manner analogous to that of PORATH[2] and GEDIN AND PORATH[9], who compensated during electrophoresis by elevating the buffer level in the vessel here containing (C). Precise control with their technique requires a colored internal marker. (c) Hours required for the movement of protein to the starting position are eliminated. (d) Column and sample are freer from troubles in circulation of buffer. The membrane isolates the liquid in the vertical buffer arm from the flow dynamics in the pumping system.

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Preliminary Notes

On the nature of the fluorescence of enzyme–DPNH complexes

The discovery by BOYER AND THEORELL[1] that the addition of a DPNH-linked enzyme to a solution of DPNH causes an increase in the intensity of fluorescence and a shift of the emission spectrum to shorter wavelengths, has provided an elegant and versatile tool for the investigation of enzyme–coenzyme complexes. However, the usefulness of such studies has been sharply limited by the lack of any generally valid and experimentally substantiated explanation of the phenomenon. Hypotheses involving changes in the structure, conformation, planarity, or electron distribution of the reduced nicotinamide ring have been widely proposed.

We have found, however, that the binding of DPNH in *any* manner which reduces the possibility of inter- or intra-molecular collisions will cause both an increase in fluorescence intensity and a shift of the emission spectrum to shorter wavelengths.

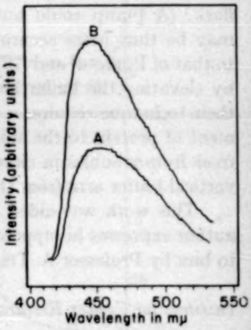

Fig. 1. The fluorescence emission spectra of DPNH and of an insoluble complex of DPNH, Zn^{++}, and phosphate. Curve A, $2 \cdot 10^{-5}$ *M* DPNH in 0.2 *M* Tris buffer, pH 7.6. Curve B, same solution after addition of $ZnSO_4$ to a final concentration of $7.5 \cdot 10^{-3}$ *M*, and sodium phosphate to a final concentration of $1.8 \cdot 10^{-3}$ *M*. Spectra were recorded with a Farrand spectrofluorometer equipped with a 1 P 21 photomultiplier tube and an Osram XBO 162 lamp, using 10-mμ slits. A quartz cuvette (1 cm × 1 cm) was used with a Corning 7–54 filter in front and a 3–72 filter behind it. The activation wavelength was 345 mμ. The fluorescence of all components other than DPNH was negligible.

Both phenomena are demonstrated in Fig. 1. The addition of zinc and phosphate ions to a solution of DPNH causes the formation of an insoluble complex. The stirred suspension shows an increase in fluorescence intensity of 92 %. Since the spectra are uncorrected for changes with wavelength of lamp emission, photomultiplier-tube sensitivity, and transmittance of filters, the exact shape of the curves has only relative meaning; however, the spectral shift has the same magnitude and direction as that caused by the addition of L-glutamic dehydrogenase to a solution of DPNH under the same instrumental conditions. These phenomena are not caused by scattered light. The identical shape of the activation spectra in the presence of and in the absence of the complex, and the unchanged fluorescence of a DPNH solution in the presence of stirred suspensions of sea sand or silica gel, prove that the optical-filter system is effectively preventing any scattered light from reaching the photomultiplier tube.

Abbreviations: DPNH, reduced diphosphopyridine nucleotide; Tris, tris(hydroxymethyl)-aminomethane.

The relative increase in fluorescence intensity when complexes are formed between DPNH and a variety of other substances are listed in Table I. It can be seen that an increase in fluorescence intensity occurs whenever the reduced coenzyme is either incorporated into a solid phase by complex formation, or is bound to the surface of a large molecule already present in suspension.

TABLE I

INCREASE IN DPNH FLUORESCENCE INTENSITY IN VARIOUS COMPLEXES

Substances added to DPNH solution	*Presence of a precipitate*	*Increase in fluorescence intensity (%)*
Zn^{++}, phosphate	+	50
Zn^{++}, phosphate, albumin	+	86
Silica gel	+	0
Mg^{++}	—	0
Mn^{++}	+	102
Dowex 1-X-4	+	34

Experimental procedures and instrumental conditions were the same as for Fig. 1. DPNH concentration was $2 \cdot 10^{-5}$ *M* in all experiments. Final concentrations of added components were: phosphate, $1.8 \cdot 10^{-3}$ *M*; Zn^{++}, Mg^{++}, and Mn^{++}, $7.5 \cdot 10^{-3}$ *M*; bovine serum albumin, 2.7 mg/ml. Fluorescence emission intensities were read at 465 mμ.

It is well established that in any group of organic molecules, fluorescence is strongest in those compounds which contain the most rigid nuclear framework, or which are, in some manner, protected from deactivating collisions[2]. The increased intensity of fluorescence of dyes adsorbed on colloids was first noticed in 1895 by WIEDEMANN AND SCHMIDT[3]. DPNH is a particularly flexible molecule possessing many degrees of freedom. Such a molecule, having reached an excited state by absorption of a quantum of light, may dissipate that energy by any of a number of competing pathways, of which fluorescent emission is only one. Of these competing pathways, only two concern us here; namely, internal conversion, and solvent quenching. If a highly flexible molecule is bound by more than one point of attachment to some relatively inflexible surface, the possibilities for internal conversion are greatly reduced, and the number of deactivating collisions with solvent molecules may be lessened.

Thus it can be seen that an increase in intensity and a shift to shorter wave lengths of the fluorescence of a reduced coenzyme caused by the addition of an enzyme, is to be interpreted as evidence *only* of the binding of the reduced coenzyme on the surface of the enzyme.

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Nature and origin of an inhibitor of oxidative phosphorylation present in freshly isolated sarcosomes from blowfly thoraces

In mitochondria from larvae of the wax-moth, *Galleria mellonella*, the stimulation of the ATP-orthophosphate exchange reaction by albumin has been shown to be due to the binding of a fat-soluble inhibitor by the albumin[1]. In experiments which will be reported elsewhere we have shown that a similar explanation can account for the stimulation by albumin of oxidative phosphorylation in sarcosomes from the thorax of the blowfly, *Calliphora erythrocephala*. Furthermore, we have been able to extract an inhibitor from freshly isolated sarcosomes with iso-octane. Thus it appeared that the inhibitor might be present in the thorax of the living insect. In order to test this possibility extracts were prepared both from thoraces which had been boiled for 1 min in iso-octane before being extracted, and from an equal number of thoraces which were ground for about 1 min before boiling iso-octane was added. The effect of these extracts on the P/O ratio obtained with fresh sarcosomes was then examined. For this purpose the sarcosomes were isolated from thoraces which were crushed in a sucrose medium to which albumin had been added to remove the inhibitor normally present in freshly isolated sarcosomes. The albumin was removed by washing and resuspending the sarcosomes in albumin-free medium. The results (Table I) show that an inhibitor is present in the crushed thoraces, and that its action is reversed by albumin. Extracts of thoraces which were boiled before being crushed did not contain the inhibitor, and therefore we conclude that it is produced by enzymic action when the tissue is crushed, and that it is not present in the living insect.

It has been suggested that when liver mitochondria are aged long-chain fatty acids are released, and that these are responsible for the low P/O ratio obtained with aged mitochondria[4, 5]. In view of this suggestion, and of the known ability of albumin

TABLE I

INHIBITION OF OXIDATIVE PHOSPHORYLATION BY EXTRACTS OF BLOWFLY THORACES AND ITS REVERSAL BY ALBUMIN

Additions	*ΔO µatoms*	*ΔP µmoles*	*P/O*
None	3.9	6.4	1.6
Iso-octane extract of boiled thoraces	3.8	6.3	1.7
Iso-octane extract of crushed thoraces	2.6	1.8	0.7
Bovine plasma albumin fraction V (1 %)	6.0	11.1	1.9
Iso-octane extract of boiled thoraces + albumin (1 %)	5.5	10.6	1.9
Iso-octane extract of crushed thoraces + albumin (1 %)	4.7	10.0	2.1

Reaction medium: sucrose, 198 µmoles; glucose, 66 µmoles; $MgCl_2$, 6.6 µmoles; Tris-HCl buffer (pH 7.3), 11 µmoles; ethylenediaminetetraacetate, 2 µmoles; ADP, 3.3 µmoles; α-ketoglutarate, 12 µmoles; hexokinase, 550 Berger units; phosphate (pH 7.3), 13 µmoles; sarcosomes (2.2 mg protein); final volume, 1 ml. 0.1 ml 10 % KOH in centre well, temp. 25°, incubation time 20 min. O_2 uptake measured with differential manometers; phosphate uptake estimated from change in orthophosphate measured by method of MARTIN AND DOTY[3]. Sarcosomes isolated, by procedure similar to that described by LEWIS AND SLATER[2], in medium (3 ml for 50 thoraces) containing 0.32 *M* sucrose, 0.01 *M* Tris buffer (pH 7.3), 0.01 *M* ethylenediaminetetraacetate, 2 % albumin, both washed (2.5 ml) and suspended (0.75 ml) in albumin-free medium. Iso-octane extract equivalent to 2 thoraces evaporated in flasks.

Abbreviations: ADP, ATP, adenosine di- and triphosphate; Tris, tris(hydroxymethyl)-aminomethane.

to bind long-chain fatty acids[6], it seemed likely that the inhibitor produced in the blowfly thoraces may be of a similar nature. The results shown in Table II confirm the acidic nature of this inhibitor, in that it is completely removed from the iso-octane extract when this is shaken with K_2CO_3 solution, and may be recovered from the latter by acidification and extraction with iso-octane or ether. This procedure effects a considerable purification of the inhibitor from non-acidic substances, particularly neutral fat. In experiments designed to establish its identity an ethereal solution of the inhibitor, partially purified as described above, was treated with diazomethane, and the methylated products were examined by gas chromatography. This revealed the presence of methyl esters of C_{14}, C_{16}, and C_{18} acids in the proportions 4:72:24.

TABLE II

REMOVAL BY ALKALI OF AN INHIBITOR OF OXIDATIVE PHOSPHORYLATION FROM ISO-OCTANE EXTRACT OF BLOWFLY THORACES

Expt.	*Additions*	*ΔO μatoms*	*ΔP μmoles*	*P/O*
1	None	3.0	3.4	1.1
	Iso-octane extract of crushed thoraces	2.0	0.4	0.2
	Iso-octane extract after shaking with K_2CO_3	2.4	3.4	1.4
2	None	2.0	4.8	2.4
	Iso-octane extract of crushed thoraces	1.9	1.6	0.8
	Iso-octane extract of acidified K_2CO_3	1.3	1.1	0.8

Reaction medium and conditions as in Table I. Iso-octane extract shaken twice with an equal volume of 1 *N* K_2CO_3, and washed with water. K_2CO_3 acidified with HCl and extracted twice with equal volume of iso-octane.

In a similar extract prepared from an equal number of boiled thoraces a trace of C_{16} acid only was detected, equivalent to < 4 % of the total long-chain acids in the inhibitory extract.

It appears then that the low P/O ratio obtained with blowfly sarcosomes isolated in the absence of albumin is due in part to the presence of free long-chain fatty acids released enzymically when the tissue is crushed.

The gas chromatography was carried out by Gas Chromatography Ltd., 176 Old Brompton Road, London.

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Solubilization and enzymic activity of bacterial cytochrome *b*

We wish to report that trypsin hydrolysis of the particulate fraction of a pyruvate oxidase system releases heat-labile, non-dialyzable, enzymically-active material into a soluble form. Since the physical and enzymic properties of cytochrome-containing particulate fractions prepared from various species of aerobic bacteria share common characteristics, the particle solubilization described in this paper may be of general use in the study of electron transport in these organisms.

In addition to the well documented pathway of pyruvate oxidation mediated by DPN, CoA and lipoic acid, *Escherichia coli*, and to a greater extent *Proteus vulgaris*, contain a pyruvate oxidase which oxidatively decarboxylates pyruvate to acetate and CO_2 with the uptake of molecular O_2 according to equation (1)[1–4].

$$CH_3{-}CO{-}COO^- + \tfrac{1}{2}\, O_2 \rightarrow CH_3{-}COO^- + CO_2 \qquad (1)$$

Ferricyanide or 2,6-dichlorophenolindophenol will substitute for O_2 as the electron acceptor for this enzyme system according to equation (2).

$$CH_3{-}CO{-}COO^- + 2Fe^{+++}(CN)_6 + H_2O \rightarrow CH_3{-}COO^- + CO_2 + 2Fe^{++}(CN)_6 + 2H^+ \qquad (2)$$

The enzyme system catalyzing both these reactions has been separated into a soluble enzyme fraction and a cytochrome-containing particulate fraction which sediments as a brownish transparent gel at high gravitational forces[3–5].

Studies in this laboratory have shown that in an acetate-requiring mutant of *E. coli* the genetic block lies in the DPN, CoA, lipoic acid pathway[6], and in this instance the mutant produces high levels of the particulate system in response to the accumulation of pyruvate in the growth medium. The soluble fraction of the mutant pyruvate oxidase system has been obtained in a high state of purity (> 90 % pure as judged from the schlieren pattern in the ultracentrifuge). The purified soluble enzyme is a non-autooxidizable flavoprotein and contains 1 mole FAD per 200,000 g protein[7]. However, the particulate fraction is not amenable to classical protein-purification procedures, and various attempts to prepare soluble protein or lipoprotein fractions from the particulate fraction using lipid solvents have led to complete or almost complete loss of enzymic activity. On the other hand, following brief exposure to trypsin approx. 50 % of the total dry weight of the particulate fraction is converted to a soluble form which is no longer sedimentable at 144,000 × *g* for 1 h. When coupled with the purified soluble flavoprotein, the soluble material prepared in this manner is enzymically active in catalyzing both reactions (1) and (2) (Fig. 1). The results shown in Fig. 1 form the basis of the assay method to measure the activity of the particulate fraction. In the presence of an excess of the soluble flavoprotein the rate of O_2 or ferricyanide reduction is proportional to the concentration of the particulate fraction or of the soluble material derived from it.

The solubilization of the particulate fraction by trypsin treatment, as indicated by a decrease in absorbancy, parallels the release of enzymic activity which is no longer sedimentable at 144,000 × *g* for 1 h. The solubilized material obtained from the trypsin-hydrolyzed particulate fraction is denatured when subjected to $(NH_4)_2SO_4$ fractionation but has been further purified by acid precipitation. Reduction of the solubilized material with $Na_2S_2O_4$ or enzymically with the soluble flavoprotein and pyruvate clearly indicate the presence of a cytochrome b_1 as evidenced by the

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Abbreviations: DPN, diphosphopyridine nucleotide; CoA, coenzyme A.

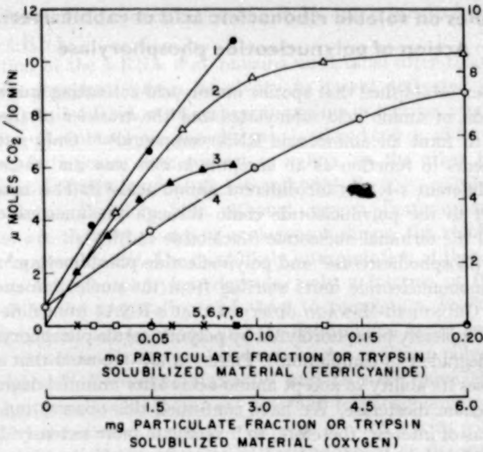

Fig. 1. The requirement for the soluble flavoprotein and the particulate fraction or soluble material derived from the particulate fraction for activity with either O_2 or ferricyanide as the electron acceptor. With ferricyanide as electron acceptor the complete system contained 100 μmoles potassium phosphate buffer, pH 6.0, 10 μmoles $MgCl_2$, 0.1 μmole thiamine pyrophosphate, 50 μmoles potassium pyruvate, 25 μmoles $K_3Fe(CN)_6$ and 0.48 mg soluble flavoprotein with increasing amounts of the particulate fraction (○—○, curve 4), or the trypsin-solubilized particulate fraction (●—●, curve 1). With O_2 as the electron acceptor the complete system contained the same components as the ferricyanide assay with ferricyanide omitted and 750 μmoles KOH in the center well to absorb CO_2. In the O_2 assay 1.45 mg of the soluble flavoprotein was added together with increasing amounts of the particulate fraction (△—△, curve 2), or the trypsin-solubilized particulate fraction (▲—▲, curve 3). Curves 5 and 6 show that the particulate fraction in the absence of the soluble flavoprotein has no activity with O_2 (□—□) or ferricyanide (■—■) as electron acceptor. Curves 7 and 8 (×—×, O_2; ◓—◓, ferricyanide) likewise show that the trypsin-solubilized fraction has no activity in the absence of the soluble flavoprotein. The ferricyanide reactions were carried out in N_2 and the rate of reaction was determined manometrically by CO_2 evolution. The O_2 assays were incubated in air. All incubations were at 30° in Warburg cups in a total volume of 1 ml for the time indicated.

appearance of reduced α and β bands at 556 and 523 mμ and a shift in the Soret band from 412 to 427 mμ. Preliminary runs in the ultracentrifuge indicate that the solubilized material is heterogeneous and is composed of two major components.

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Studies on soluble ribonucleic acid of rabbit liver. Action of polynucleotide phosphorylase

It has recently been established that specific amino-acid-activating enzymes catalyze both the formation of amino acid adenylates and the transfer of the amino-acyl moiety to RNA to form an amino acid-RNA compound[1,2]. Only cytoplasmic or soluble RNA appears to function as an acceptor in this way and there is evidence that there exist different *s*-RNAs for different amino acids[2–5]. The amino acid presumably is bound to the polynucleotide chain through the unesterified 2′- or 3′-hydroxyl group of the terminal nucleotide (nucleoside end)[3,6,7].

Both venom phosphodiesterase[8] and polynucleotide phosphorylase[9] act by stepwise removal of mononucleotide units starting from the nucleoside end of a polynucleotide chain. GRUNBERG-MANAGO observed that *s*-RNAs from different sources are slowly and incompletely phosphorolyzed by polynucleotide phosphorylase whereas other RNAs are degraded to completion[10]. PREISS *et al.*[3] reported that *s*-RNA from *Escherichia coli* loses its ability to accept amino acids after minimal degradation (less than 5 %) with venom diesterase. We have confirmed this observation with rabbit liver *s*-RNA. It was of interest, therefore, to investigate more extensively the action of polynucleotide phosphorylase on *s*-RNA, particularly with respect to its effect on amino acid acceptor ability.

As shown in Table I the phosphorolysis of rabbit liver *s*-RNA[*] stops at 20–30 % of completion. The cessation of the reaction is not caused by enzyme inactivation or inhibition since (1) addition of fresh enzyme after 5 h produces no additional breakdown; (2) polyadenylic acid[11] added after 5 h is rapidly phosphorolyzed; and (3) the acid-soluble reaction products are not inhibitory. The extent of phosphorolysis

TABLE I

PHOSPHOROLYSIS OF *s*-RNA

Expt.	*Time* *h*	*µmole* $^{32}P_i$ *incorporated*	% *phosphorolyzed*
A	0	0.00	—
	5	0.12	28
	6	0.12	28
B	3	0.09	16
	6	0.11	20
	22	0.10	18

The reaction mixtures (0.20 ml) contained 0.1 *M* tris(hydroxymethyl)aminomethane buffer, pH 8.2, 10 m*M* $MgCl_2$, 0.2 m*M* ethylenediaminetetraacetate and potassium [^{32}P]phosphate buffer, pH 7.4 (23 m*M* in Expt. A and 10 m*M* in Expt. B). The amounts of *s*-RNA, in µmole organic P, were 0.43 and 0.56 in Expts. A and B respectively. 90 and 210 µg of a preparation of *A. agilis* polynucleotide phosphorylase of specific activity 23 (ref. 11) were used in Expts. A and B respectively. After incubation at 37°, 0.05 ml aliquots were added to 1.00 ml cold 2.5 % $HClO_4$ and the precipitate was removed by centrifugation. The supernatant fluids were treated with charcoal to determine ^{32}P in acid-soluble nucleotides[12]. The results are given as µmole $^{32}P_i$ incorporated into acid-soluble nucleotides per 0.2 ml reaction mixture.

Abbreviations: RNA, ribonucleic acid; *s*-RNA, RNA from the soluble portion of a tissue homogenate; P_i, inorganic orthophosphate.

[*] The *s*-RNA used in these experiments, prepared from rabbit liver by NaCl extraction, contained less than 0.01 µmole of bound amino acids per 25 µmoles of *s*-RNA ribose.

is not dependent on the concentration of *s*-RNA and is unaffected by mild alkaline treatment of *s*-RNA.

The portion of the *s*-RNA that remains undigested after treatment with polynucleotide phosphorylase retains its ability to accept activated amino acids. The specific activity[*] of *s*-RNA, with respect to proline or leucine, was exactly the same before and after phosphorolysis, namely, 914 ± 58 and 906 ± 48 for proline, 140 ± 20 and 128 ± 24 for leucine; with respect to valine, on the other hand, the specific activity showed a small decrease, 96 ± 6 and 76 ± 8.

It is apparent, therefore, that although venom diesterase and polynucleotide phosphorylase are thought to act in analogous fashions, the results of their action on *s*-RNA are quite different. The most likely interpretation of these findings suggests that polynucleotide phosphorylase degrades only a fraction of the polynucleotide chains present in the preparation and these to completion. 70–80 % of the chains may be entirely resistant to attack and the determining factor could be the extent of secondary structure (hydrogen bonding) in the resistant polynucleotide chains. The experiments of GRUNBERG-MANAGO[10] and OCHOA[13] have shown that multistranded polyribonucleotide chains are resistant to phosphorolysis. This hypothesis is supported by the observation that treatment of *s*-RNA with polynucleotide phosphorylase has essentially no effect on either the modal or the weight-average sedimentation coefficient (Table II).

TABLE II

EFFECT OF POLYNUCLEOTIDE-PHOSPHORYLASE TREATMENT ON SEDIMENTATION OF *s*-RNA[*]

	Sedimentation coefficient	
	Weight average	*Mode*
s-RNA (untreated)	4.0	4.0
s-RNA (undigested)	3.9	4.1

[*] The absorbancy of the *s*-RNA solution was 1.0.
Measurements at 59,780 rev./min were made in 0.2 *M* NaCl, using the Spinco Model E Ultracentrifuge with u.v. optics. Values were computed from tracings obtained from the photographic images using the Spinco Model RA Analytrol, equipped with a microanalyzer attachment[14].

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[*] Specific activity is expressed as counts/min/absorbancy in a hot-$HClO_4$ digest of *s*-RNA.

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Peptides isolated from peptic hydrolysate of diisopropylphosphoryl-trypsin

In the comparative studies on protein structures[1] which our laboratory has been working on we have been concerned above all with chymotrypsinogen and trypsin. A survey of the peptide sequences in these proteins so far known was published by us earlier[2].

The present paper gives a preliminary report on results obtained by studying peptides isolated from a peptic hydrolysate of DIP-trypsin. This study permits us to ascertain mutual bonds in the vicinity of the residues of lysine and arginine, which are split on tryptic hydrolysis.

DIP-trypsin (1000 mg) was hydrolyzed by pepsin (enzyme to substrate ratio, 1:50) at 37° and pH 2.0 for 2.5 h. A small quantity of non-dialyzing material was removed from the peptic hydrolysate by dialysis against distilled water. The freeze-dried dialysate was fractionated on a Zerolit 225 X2 ion-exchange resin (100–200 mesh) column of 2.2 × 150 cm at 40° using an elution gradient. For this purpose volatile buffers composed of pyridine and formic acid or pyridine and acetic acid proved satisfactory as in the case of the separation of a peptic hydrolysate of chymotrypsinogen[3].

Elution was initiated with the 0.1 *M* buffer (with respect to pyridine) and the gradient developed by gradually introducing solutions of increasing molarity and pH-value into a mixing vessel (volume, 2200 ml). The solutions used were as follows: 0.2 *M*, pH 3.1; 1 *M*, pH 5.1; 2 *M*, pH 5.2; 2 *M*, pH 7.0 and 2 *M* ammonia. The eluates were collected in 20-ml fractions at a flow rate of 1 ml/min.

The course of the separation on the column was checked by submitting dry residues of 0.4-ml aliquots taken from each fraction to descending paper chromatography[3] in the system *n*-butanol–pyridine–acetic acid–water (15:10:3:12)[4]. According to the chromatograms, individual fractions obtained from the column were pooled and fractionated further by preparative chromatography on Whatman No. 1 or No. 3 paper in the system[4] mentioned above. The zones obtained were purified by paper electrophoresis in the system formic acid–acetic acid of pH 1.9 at a potential gradient of 65 V/cm.

A majority of the zones isolated comprised individual peptides as was proved by calculating the molar ratios of amino acids by comparing the peptide hydrolysate

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Abbreviation: DIP-, diisopropylphosphoryl-.

with a set of different concentrations of standard samples, and by determining the N- and C-terminal groups by means of the dinitrophenylation technique and hydrazinolysis, respectively. The partial structure and/or the complete amino acid sequence of the peptides has been determined.

Table I presents a survey of peptides in order of emergence from the column.

TABLE I

PEPTIDES ISOLATED FROM A PEPTIC HYDROLYSATE OF DIP-TRYPSIN

AspNH$_2$·Ser
Val·(AspNH$_2$, Glu$_2$, Gly)
Val·(Ala, Ser)·Ser
Ileu·(Ser, AspNH$_2$)·GluNH$_2$
AspNH$_2$·Ileu
Ileu·(AspNH$_2$, Ser)
Ileu·(Ala, Ser)
Asp·(Ala, Ileu)·Ser
Val·(Leu, Ser)
Ileu·(Ser, Leu)
Asp·(Ser$_2$, Tyr)
(I)Leu·(Ser, (II)Leu)
Pro·(Leu, Ileu)
(Lys, Ser$_2$, Ala, Ileu)
Lys·(AspNH$_2$, Ser, Ala, Leu)·Ser
Lys·(Ser$_2$, Glu, Thr, Ala, Ileu)·Asp
Lys·(Ser, Ala)
AspNH$_2$·Ser·Arg·Val·Ala·Ser
Val·(Arg, Leu, Gly, Glu, Asp, (I)Leu, AspNH$_2$)
Val·Arg·Leu·(Gly, GluNH$_2$)·Asp
Arg·Val·Ala·Ser
Ileu·(AspNH$_2$, Ser, Glu, Try)
(Leu, Lys)·Leu
Val·(Arg, Leu, Gly)
Ileu·(Ser, Gly)·Try·Gly·(AspNH$_2$, Thr)·Lys·(Ser$_3$, Gly, Thr, Pro, Tyr, Val, AspNH$_2$)
Tyr·(Ser, Val$_2$, Try)
Ileu·(His, Lys$_2$, Ser$_4$, Ala, Pro, Tyr)
Ileu·(Ser, Gly)·Try·Gly·(AspNH$_2$, Thr)·Lys
(Lys, Ser, Ala, Leu)

It can be seen from Table I that, among others, peptides containing lysine and arginine residues in the middle of the chain were isolated from the peptic hydrolysate. Both molecules of arginine, which are present in the molecule of DIP-trypsin, were revealed in the peptides isolated and their sequence established. Of the 3 molecules of histidine we succeeded in finding only one and of the 14 molecules of lysine only 8.

In addition, 3 residues of tryptophan, to which only minor attention has been paid in previous studies, were found in the peptides isolated.

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Présence de deux vasopressines dans la neurohypophyse du poulet

A partir de la post-hypophyse des mammifères, il est possible d'isoler un complexe renfermant les deux peptides *ocytocine* et *vasopressine* et une protéine inerte, la *neurophysine*[1]; un procédé de purification simultanée des deux hormones prenant

avantage de cette association particulière a été décrit[2]. Nous avons constaté récemment qu'il était possible de reconstituer le complexe à partir d'ocytocine, de vasopressine et de neurophysine provenant d'espèces différentes[3]. La neurohypophyse du poulet contenant des quantités appréciables d'ocytocine et de vasopressine mais peu ou pas de neurophysine, la purification des hormones a pu cependant être réalisée en effectuant un apport de neurophysine de cheval, et en appliquant ensuite le procédé habituel[4].

L'extraction de la poudre acétonique neurohypophysaire* (0.4 unité U.S.P. ocytocique ou vasopressique/mg) par H_2SO_4 dilué, l'addition de neurophysine de cheval, et la précipitation du complexe par NaCl sont effectuées dans les conditions antérieurement décrites[4]. Le complexe est dissocié par l'acide trichloracétique à 5 % qui précipite la neurophysine; on trouve dans le liquide surnageant 75–85 % des deux activités initiales. Après élimination de l'acide trichloracétique par passage sur une colonne d'Amberlite IR-45, les hormones sont chromatographiées sur une colonne d'Amberlite IRC-50 en utilisant un gradient de pH et de force ionique au moyen de tampons acétate d'ammonium. Des fractions de 1 ml sont recueillies et on dose les activités ocytocique et vasopressique dans ces fractions. La Fig. 1 indique les résultats.

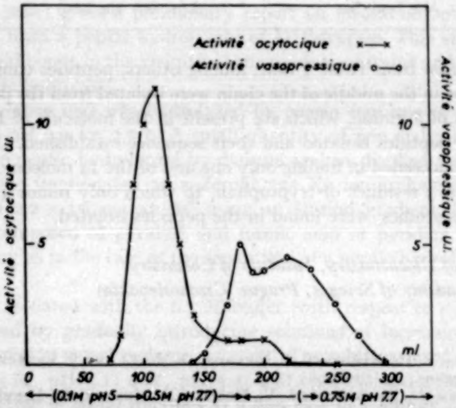

Fig. 1. Chromatographie des hormones neurohypophysaires du poulet sur Amberlite IRC-50: les hormones sont adsorbées au sommet d'une colonne de résine forme H (0.9×10 cm); la colonne est ensuite équilibrée avec un tampon acétate d'ammonium 0.1 *M* à pH 5.0 et le gradient est successivement réalisé avec des solutions 0.5 *M* et 0.75 *M* à pH 7.7.

80 % de l'activité ocytocique soumise à la chromatographie se trouve dans un pic qui occupe la position habituelle de l'ocytocine des mammifères; à ce pic correspond une substance unique qui possède la composition en acides aminés de l'ocytocine des mammifères[4]. L'activité vasopressique se trouve répartie en deux pics correspondants à deux substances: la première sortie de la colonne (vasopressine I) occupe une

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position inhabituelle mais la deuxième (vasopressine II) occupe la position de l'arginine-vasopressine des mammifères. Les tubes 170–200, contenant de la vasopressine I et les tubes 240–300, contenant la vasopressine II sont rassemblés, puis après élimination de l'acétate d'ammonium, les substances sont hydrolysées par HCl 6 *N*.

La vasopressine II possède la composition en acides aminés de l'arginine-vasopressine. La vasopressine I possède une composition voisine, mais le résidu de phénylalanine est remplacé par un résidu d'isoleucine. Cette nouvelle vasopressine possède une composition identique à celle d'un peptide synthétique, l'*arginine-vasotocine*, synthétisé par Katsoyannis et Du Vigneaud[5]; comme ce dernier peptide, la nouvelle vasopressine possède une activité ocytocique secondaire relativement élevée si on la compare à l'activité vasopressique.

Cependant, une comparaison soigneuse des deux produits est nécessaire avant de conclure à une identité. Quoiqu'il en soit, la neurophysine du poulet, à la différence de celle des mammifères, contient 3 hormones neurohypophysaires de composition voisine.

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The effect of paramagnetic substances on the conversion of some pyrimidines by ultraviolet radiation

In previous papers[1–6] we have reported upon the effects of ultraviolet radiation (2537 Å) on some components of nucleic acids in aqueous solution. A reversible hydration of uracil was known from the work of Sinsheimer and Hastings[7]. Moreover, long-continued irradiation leads to an irreversible destruction of these compounds. These reactions could be followed by the alterations of the ultraviolet spectra. We have found that besides these two photochemical phenomena, the pyrimidines show another reaction which is probably the most important. It appears that irradiation of aqueous solutions of the compounds, without any time lag, also brings about partial conversion into low-absorbing substances. The probable connection between this photochemical conversion and the lethal and mutagenic effects of ultraviolet radiation on micro-organisms has been suggested[1].

This reaction, which we have called "the first irreversible reaction" is remarkable because the extent to which it occurs appears limited to a specific percentage of the amount of the pyrimidine originally present (uracil, 6 %; thymine, 2 %; orotic acid, 13 %; cytosine, 3 %).

We have recently found a relationship between the amount of conversion and the oxygen concentration. Irradiation of an aq. solution of uracil saturated with O_2 brings

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about a 3 % conversion, whereas expulsion of this gas, by bubbling through N_2 or H_2, raises this percentage to 9–10 %. This oxygen effect is still more pronounced in the case of orotic acid (in O_2-saturated solution, 7 %; after expulsion of O_2, 35–37 %).

The occurrence of an equilibrium between orotic acid and its irradiation product could be demonstrated by the following experiments. If orotic acid is first irradiated for a short time in O_2-free solution the absorbancy decreases about 35 %; saturating the solution with O_2 and repeating the irradiation causes a sharp rise of the absorbancy until a maximum is reached equal to about 7 % conversion of the orotic acid[5]. From these and other experiments[5] we must conclude that the so called "first irreversible reaction" is by no means irreversible.

This remarkable effect of O_2 was supposed by us to be due to its paramagnetism[4]. If this assumption is correct it might be expected that all paramagnetic compounds will exert an effect on "the first irreversible reaction" similar to that of O_2. This could be confirmed. Without any exception all paramagnetic ions tested (Cu^{++}, Co^{++}, Ni^{++}, Cr^{+++}, Mn^{++} and Fe^{++}) have shown the same effect on the equilibrium of "the first irreversible reaction" as O_2. The experiments were performed in acid milieu to prevent salt formation. A solution of known concentration of orotic acid in 0.2 *N* HCl was saturated with N_2 and irradiated for 15 min. After addition of the metal-salt solution to a final concentration of $5 \cdot 10^{-3}$ *M*, the irradiation was repeated for 5 min. The paramagnetic ions brought about a partial recovery of the orotic acid, which had disappeared as a result of the first irradiation (orotic acid, 35–37 % decrease in absorbancy to 10–17 % decrease). Diamagnetic ions on the other hand (K^+, Al^{+++}, Ca^{++}, Mg^{++}, Cd^{++} and Zn^{++}) were completely inert, the percentage converted in the first irradiation (35–37 %) remaining unchanged.

A quantitative relation between the concentration of a paramagnetic substance and the percentage of reversion was studied with Ni^{++}. Concentrations of $5 \cdot 10^{-3}$, $2.5 \cdot 10^{-3}$ and 10^{-3} *M* caused a rise in the absorbancy of the solutions corresponding to a reversion of the low-absorbing product from 35 % to 10 %, 16 % and 24 %, respectively.

Experiments similar to those described with orotic acid were also performed with uracil and generally the same results were obtained. However, no recovery of uracil could be found if the time between the two irradiations was too long. It is assumed that the reaction product of uracil is rather unstable. Some arguments can be given for this supposition.

The experiments with orotic acid strongly support our assumption that the rapid, seemingly irreversible, reactions lead to an equilibrium condition appropriate to the conditions of the ultraviolet irradiation. As soon as the irradiation is stopped this equilibrium is frozen. Changing of the conditions, *e.g.* by adding paramagnetic substances, results in a shift of the equilibrium under renewed irradiation. A reaction on a triplet level was supposed in one of our previous papers[5]. Singlet-triplet transitions are facilitated by paramagnetic molecules or ions. This will result in a lower concentration of the pyrimidine in the active form (triplet state) and consequently in a smaller amount of the irradiation product.

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